STUDIES IN THE IMMUNE CONTROL OF HERPES SIMPLEX VIRUS TYPE 1 (HSV-1) LATENCY: THE ROLE OF $CD4^+$ T CELLS AND IL-10 IN HSV-1 LATENCY

by

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Submitted to the Graduate Faculty of

School of medicine in partial fulfillment

of the requirements for the degree of

Master of Science

University of Pittsburgh

2015

UNIVERSITY OF PITTSBURGH

SCHOOL OF MEDICINE

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STUDIES IN THE IMMUNE CONTROL OF HERPES SIMPLEX VIRUS TYPE 1 (HSV-1) LATENCY: THE ROLE OF CD4$^+$ T CELLS AND IL-10 IN HSV-1 LATENCY

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Herpes simplex virus 1 (HSV-1) infection of the eye results in lytic viral replication in the corneal epithelium followed by a quiescent infection of the sensory neurons of the trigeminal ganglia (TG), which innervate the cornea. Reactivation of the HSV-1 from this latent state can result in pathologies, such as stromal keratitis in the eye and viral encephalitis. Currently, no therapeutics are available that eliminate latent HSV-1 infection or completely inhibit viral reactivation events. Development of more effective therapeutics to prevent HSV-1 reactivation and recurrent disease has been hindered by a lack of understanding of the immune mechanisms involved in maintaining viral latency and how they are controlled. CD8$^+$ T cells are known to play multiple direct roles in preventing viral reactivation during latent HSV-1 infection, but a functional role for CD4$^+$ T cells has not been established. **Our current hypothesis is that CD4$^+$ T cells present in the latent HSV-1-infected TG are a virus-specific, locally-activated population that regulates CD8$^+$ T cell function through secretion of cytokines, such as IL-10 and IFN-γ, thereby influencing HSV-1 latency.** Here, we provide data indicating that at least a portion of the CD4$^+$ T cells in the TG during latency are HSV-specific and that they localize to defined APC populations in the latently infected TG. Our data also indicate that the numbers and functionality of CD8$^+$ T cells that are specific for subdominant HSV-1 epitopes are regulated at least in part, by CD4$^+$ T cells, through an IL-10-dependent mechanism.

TABLE OF CONTENTS

LIST OF FIGURES

ACKNOWLEDGEMENT

I would first like to thank my committee for taking the time to review my thesis. Thank you to my mentor, Dr. Bob Hendricks, for giving me the opportunity to be a member of his dedicated laboratory team. Thank you for your patience and guidance over the last few years and for the wonderful experimental suggestions you have provided to help guide my work and professional career.

I would also like to thank current and former members of the laboratory who have helped acclimate me to the lab and have helped on so many occasions with experimental design, execution, and data interpretation: Hongmin Yun, Alex Rowe, Moira Geary, Kate Carroll, K-Ann Buela, and Sophia Jeon.

Thank you to the primary investigators, core technicians, and members of other laboratories in the Ophthalmology department that have aided me during my time here by helping solve experimental problems and providing me reagents and advice when I was in need or simply by giving me a laugh when I was having a rough day in the lab.

To the Ophthalmology department administrative staff, thank you for helping me keep my seminar schedule organized and for answering the endless numbers of questions I seem to always have for you. To the many wonderful friends I have made here in Pittsburgh, thank you for the many laughs, tears, and commiserating events.

Thank you to my mother and father, who have always encouraged me to pursue the things that make me happy in life. Thank you to my grandmother for believing I am the greatest grandkid around even when I mess up. Thank you to my entire family for your support and encouragement from afar.

Finally, thank you to my wonderful husband, Kyle Thomas, for your unending sacrifice, patience, love and support over the last few years. Thank you for upending your life to come with me to Pittsburgh so I could pursue my dreams in science. I surely would not have been able to come this far without you by my side.

1.0 INTRODUCTION

1.1 HERPES SIMPLEX VIRUS TYPE I (HSV-1)

1.1.1 Epidemiology and Pathogenesis

Herpes Simplex Virus 1 (HSV-1) is a neurotropic viral pathogen that infects and resides within its host for the duration of the host's lifetime. While humans are its natural host, HSV-1 exhibits broad infectivity for numerous mammalian species including mice, guinea pigs, and rabbits. The infectivity of HSV-1 in human populations is evidenced by the high percentages of seropositive individuals worldwide. In the United States, greater than 50% of adults 18 years and older are seropositive for HSV-1 and that percentage increases to over 90% in adults in the 60 and older population [1-3]. Globally it is estimated that 80% of adults are infected with HSV-1 with seroprevalence increasing to 98% in some regions [4-6]. Many seropositive individuals are asymptomatic and do not experience pathologies commonly associated with orofacial HSV-1 infection, such as cold sores or inflammatory keratitis events. However, a small but significant proportion of infected individuals manifest severe pathologies, including viral encephalitis and blinding stromal keratitis. Annually, the eyesight of nearly 4,000 patients is sight impaired due to stromal keratitis and HSV-1-induced keratitis is the leading infectious cause of blindness in the United States [7, 8].

Ocular infection of HSV-1 begins with a primary lytic infection phase within the corneal epithelium in which the HSV-1 genome is replicated and progeny virus is produced. During primary infection HSV-1 infects the sensory neurons innervating the cornea and travels to the trigeminal ganglia (TG) via retrograde transport [9]. Once in the TG, retention of the HSV-1 genome in a non-replicative state within the neuronal nuclei establishes a non-lytic state known as latency [10]. Reactivation of HSV-1 from latent infection frequently results in anterograde transport of the virus to the eye followed by HSV-1 replication and shedding of progeny virus at the corneal surface [11, 12]. This release of progeny virus can be asymptomatic or can cause epithelial keratitis or HSV endotheliitis, which is clinically characterized by painful, red, watery eyes and blurred vision [11, 13]. A combination of increased vascularization and inflammation in the cornea, sensory nerve damage, and corneal desiccation are characteristic of the progression of epithelial keratitis to a more severe state of eye disease known as Herpes Stromal Keratitis (HSK). HSK is characterized by corneal opacity, edema, and severe neovascularization of and a pronounced immune infiltrate within the stroma [14]. A representative image of HSK in a C57BL/6 mouse eye is shown in **Figure 1**. HSK events can cause variable amounts of scarring of the cornea and when recurrent, often cause blindness in the infected eye [13, 14]. The molecular causes of HSV-1 reactivation remain unclear, but physical, hormonal, and psychological stress are associated with reactivation in humans and have been shown to cause reactivation in mouse models of HSV-1 infection [15-19].

There is currently no vaccine to prevent HSV-1 infection or reactivation from latency [11, 20, 21] and antiviral therapeutics are predominantly limited to DNA synthesis inhibitors that selectively impede viral replication, such as acyclovir [22]. Given the high

seroprevalence of HSV-1 and the relatively high rates of blindness caused by complications associated with ocular HSV-1 infection, the need for improved anti-HSV-1 therapeutics is apparent.

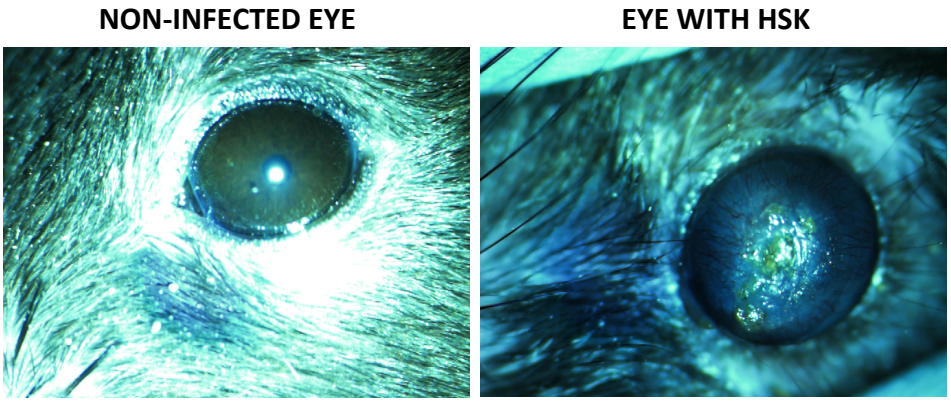

Figure 1. HSK in C57BL/6 mice.

Image in left panel shows the eye of a healthy, non-infected C57BL/6 mouse. Image on right shows HSK in the eye of a C57BL/6 mouse following infection with $1X10^5$ PFU HSV-1-RE virus. Blood vessels have infiltrated the normally avascular cornea and a dessication-induced epithelial lesion is visible. Figure graciously provided by Hongmin Yun, M.D. Eye and Ear Institute, University of Pittsburgh Medical Center, Pittsburgh, PA.

1.1.2 HSV-1 Classification, Structure, and Lytic Replication Cycle

HSV-1 is a double-stranded DNA virus or a type I virus by the Baltimore Classification system. HSV-1 is a member of the *Herpesviridae* family of viruses and is further classified as a member of the neurotropic *Alphaherpesvirinae* subfamily along with Herpes Simplex Virus Type II (HSV-2), Varicella Zoster Virus (VZV), and other members of the *Mardivirus* and *Iltovirus* genera [11]. The HSV-1 virion is comprised of 4 major structurally distinct components **(Figure 2)**. The innermost component is the double-stranded DNA genome, which is 152kb in length and encodes 84 proteins [10]. Encasing the viral genome is the

capsid, an icosahedral structure composed of viral proteins that serves to protect the DNA. Surrounding the capsid shell is the tegument, which contains various viral proteins needed for HSV-1 to effectively infect and manipulate the host cell to promote viral replication [10, 11]. The final component of HSV-1 is the envelope; a lipid bilayer of host cell origin that surrounds the tegument and contains numerous viral glycoproteins required for viral egress and target cell entry [10, 11, 23].

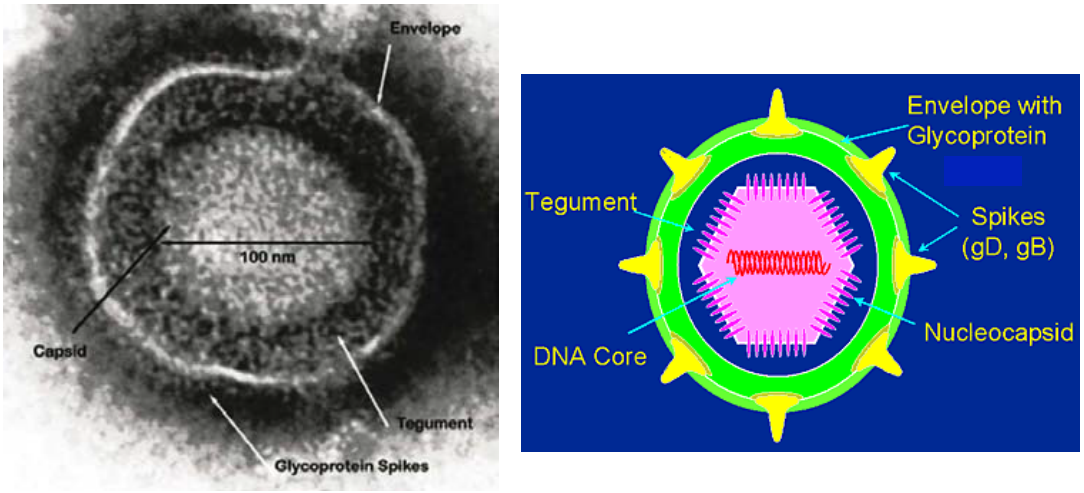

Figure 2. Structure of the HSV-1 virion.

Image in left panel: electron micrograph of an HSV-1 particle. Right panel: representative illustration of an HSV-1 particle. Both are labeled to show the 4 distinctive elements comprising the HSV-1 virus. Figure adapted from online lectures from Todar, K (2009) and Leone, P, M.D.

HSV-1 enters its target cell via a fusion event mediated by HSV-1 glycoproteins (gB, gC, gD, and the gH/gL complex) binding to specific host cell surface markers and inducing a fusion event or by endocytosis[24-26]. Once inside the cell the viral capsid traffics to the nucleus and the HSV-1 genome is deposited inside. Viral gene expression ensues to

generate proteins required for genome replication. Once genome replication is inititated, structural proteins are generated and viral capsids are assembled within the nucleus. The capsids pass through the nuclear membrane before entering the Golgi Network, where they acquire tegument proteins as well as their lipid bilayer. They completed viral particle then escapes the cell, compromising the cell membrane and inducing lysis and host cell death. The progeny virus will continue this lytic replication pattern in additional epithelial cells and will infect innervating neuronal cells. The primary infection is cleared by the innate immune system within 4-5 days. Following clearance of the primary infection in the cornea, HSV-1 establishes a latent and non-lytic stage of infection in the nuclei of neurons within the TG.

1.2 HSV-1 LATENCY

1.2.1 HSV-1 Gene Expression During Latency

Upon the establishment of latency in the sensory ganglia, lytic gene expression is silenced and no viral particle production is observed [9]. The HSV-1 genome is retained within the nuclei of infected sensory neurons as an episomal structure that is associated with cellular histones and is highly heterochromatic [27,28]. Latency Associated Transcripts (LATs) are the only *readily* detectable gene products expressed during latent HSV-1 infections. LATs are non-5'-capped, non-polyadenylated RNA transcripts that are not translated into functional proteins. Unlike lytic genes of the HSV-1 genome, the LAT locus is highly acetylated and organized into euchromatin during latency, which favors it expression [29]. LATs are present in humans and in animals latently infected with HSV-1 [30-33] and have

long been the focus of studies aimed at understanding the molecular mechanisms of HSV-1 latency.

Until recently, it was believed that there was no HSV-1 lytic gene expression during latent infection. Indeed, researchers have yet to successfully isolate any HSV-1 protein product from the latently infected mouse or human sensory ganglia. However, transcripts for the HSV-1 beta (β) genes, Infected Cell Proteins 0 and 4 (ICP0, ICP4), have been detected in human TGs harboring latent HSV-1 infection [34-36] and these levels are increased in the absence of LAT expression [37]. The discovery of HSV-1 alpha (α) gene products in latently infected human TGs calls into question the generally accepted paradigm that LATs are the only HSV-1 gene product to be expressed during latency and indicates that low levels of lytic gene expression may also occur. This hypothesis is supported by the observation that HSV-specific $CD8^+$ T cells present in the latently infected TG preferentially surround neurons positive for ICP0 and LAT transcripts when compared to neurons positive for LAT alone [36]. However, positive identification of lytic transcripts in the infected TG may simply indicate that a reactivation event is ensuing and that latency has been compromised. More studies are needed to determine if HSV-1 protein products are generated during latency and, if so, how this expression influences cellular and viral factors that maintain quiescence.

1.2.2 Immune Control of Latent HSV-1 Infection

Latent HSV-1 infection has long been viewed as a silent infection that is largely ignored by the immune system. However, this view is shifting as data published in the last 2 decades indicate that latent HSV-1 infection is dynamic. Numerous lytic genes are expressed in

addition to LATs during latent HSV-1 infection [33, 38, 39] and cells of the immune system are present and active in preventing viral reactivation in the TG [39-44]. The evidence for persistent HSV-1 lytic gene expression and immune presence in the TG combined with recently published data show high levels of exhaustion in some epitope-specific populations of $CD8^+$ T cells in the latently infected TG have prompted the shift from a view of HSV-1 latency as a quiet infection to that of a persistent or chronic infection [39, 45, 46]. This has far-reaching implications on how best to approach studies of immune function in HSV-1 latency.

1.2.2.1 Maintenance of Viral Latency by CD8+ T Cells. $CD8^+$ T cells have been most studied in mouse model systems of latent HSV-1 infection and have thus far been implicated as the primary cells involved in preventing HSV-1 reactivation [41, 42, 44]. All $CD8^+$ T cells present in the TG during HSV-1 latency are HSV-specific and surround infected neurons in clusters [38, 43, 54]. Expression of interferon-gamma (IFN-γ) by $CD8^+$ T cells has been shown to directly inhibit HSV-1 reactivation *ex vivo* by preventing transcription of the viral gene encoding ICP0 [41]. Additionally, $CD8^+$ T cells secrete non-cytotoxic Granzyme B (GrzB), which directly cleaves ICP4, a viral protein required for transcription of early and late HSV-1 genes [42]. The antigen specificity of TG-resident $CD8^+$ T cells during lytic and latent infection is varied across 19 epitopes with 50% of $CD8^+$ T cells recognizing a dominant Glycoprotein B (gB) epitope and the other 50% responding to one of 18 subdominant epitopes derived from 11 unique HSV-1 proteins [40, 45]. Further examination of epitope-specific $CD8^+$ T cell populations show a discrepancy in the capability of different HSV-specific populations in the TG to prevent viral reactivation that is dependent, in part, upon signaling through the IL-10 receptor [45]. Clear roles for HSV-

specific $CD8^+$ T cells have been defined in latent HSV-1 infection in B6 mice, but the means by which $CD8^+$ T cell effector activities are regulated are not fully understood.

1.2.2.2 CD4+ T Cells in Primary and Latent Infection. $CD4^+$ T cell functions during primary viral infections are often critical for the development of an effective adaptive immune response. IFN-γ secreted by $CD4^+$ T cells is required to induce the expression of chemokines that promote activated $CD8^+$ T cell migration into infected tissues [47]. $CD4^+$ T cell-secreted IL-2 is required for $CD8^+$ T cell migration during immune priming following LCMV infection and for CD8 memory responses in chicken ovalbumin models of T cell function [48, 49]. $CD4^+$ T cells also enhance the expression of costimulatory molecules on the surface of dendritic cells (DCs) to promote CD8 and CD4 lymphocyte activation [50, 51]. In numerous models of infection, a functional tissue-resident $CD8^+$ T cell memory population is not generated without the help of $CD4^+$ T cells early in infection [47-51] and HSV-1 infection is no exception [52]. This highlights the importance of $CD4^+$ T cell function for T cell priming and differentiation during the primary infection but $CD4^+$ T cell functions may be critical for immune regulation during chronic infection as well. During chronic lymphocytic choriomeningitis virus (LCMV) infection, $CD4^+$ T cells are believed to be the key producers of IL-21, a cytokine that prevents functional $CD8^+$ T cell exhaustion [53-54]. While IL-21 production has yet to be assessed in latent HSV-1 infection, evidence of high levels of exhaustion in $CD8^+$ T cells in the TG during latent infection suggests this cytokine is not being produced or is being in produced at suboptimal levels [40,45].

It is unclear if $CD4^+$ T cells present in the TG directly prevent viral reactivation but there is evidence for indirect $CD4^+$ T cell control of HSV-1 latency through regulation of $CD8^+$ T cell function. Indeed, 20% of $CD4^+$ T cells in the latent HSV-1-infected B6 TG express IL-10

and IL-10 appears to regulate the functionality of subdominant epitope-specific $CD8^+$ T cells [45]. It is also feasible that $CD4^+$ T cells may act directly to modulate HSV-1 latency in the TG through secretion of IFN-γ, as is observed for $CD4^+$ T cells in chronic γ-Herpesvirus 68 (γHV68) infections [55, 56] and $CD8^+$ T cells in HSV-1 infection [44]. $CD4^+$ T cells have also been observed to take on cytolytic effector functions such as FasL expression and GrzB degranulation in murine models of chronic infection [57, 58], though there is not yet evidence indicating that this occurs in latent HSV-1 infection. The role of $CD4^+$ T cells in maintaining HSV-1 latency remains unclear and while we hypothesize that $CD4^+$ T cells indirectly regulate viral latency through mediation of $CD8^+$ T cell function, it is not unreasonable to hypothesize that $CD4^+$ T cells may also have direct roles in preventing HSV-1 reactivation.

A portion of the $CD4^+$ T cells in the TG following HSV-1 infection in humans and mice are antigen specific [38, 58], indicating that effector functions elicited by these cells are likely in response to viral antigen. However, antigen non-specific $CD4^+$ T cells, particularly of the natural regulatory T (nT_{reg}) lineage, are observed to mediate immune activity during viral infection after TLR-2 and TLR-4 stimulation by viral pathogen-associated molecular patterns (PAMPs) [59, 60]. Given that the lineage and cytokine production profiles of $CD4^+$ T cells in the latently infected TG have not yet been characterized, it is unclear if these cells are acting in direct response to viral antigen presented via MHC class II molecules on the surface of antigen presenting cells (APCs) or if they are nonspecific responders to infection. Since the identification of the HSV-1-specific $CD8^+$ T cell repertoire following HSV-1 infection was realized, the functional capacity of these cells has been better defined, as described above [40,45]. Progress in defining the functional role(s) of $CD4^+$ T cells in the

modulation HSV-1 latency may also be possible through the identification of the antigen-specific $CD4^+$ T cell repertoire.

1.2.2.3 Antigen Presentation in the Trigeminal Ganglia (TG). The contributions of APCs in the maintenance of CD4 and CD8 lymphocyte function in the TG during latent HSV-1 infection have yet to be elucidated. However, the role of DCs in antigen presentation and activation of CD4 and CD8 lymphocytes in the spleen and lymph node after primary HSV-1 infection is well established [61-66]. It is also apparent that DCs present in previously infected non-lymphoid tissue are capable of presenting antigen to initiate robust responses from effector and memory T lymphocytes in numerous models of viral infection including HSV-1 [63, 66-68], indicating the potential of DC populations in the TG to present antigen to memory T cell populations during latency. Macrophages are also required for the development of effective innate and adaptive immune responses during primary HSV-1 infection. Cytokines such as IL-12 and IFN-γ are produced by macrophages early during primary HSV-1 infection to aid in the activation of the innate and adaptive immune responses [69, 70]. Macrophages are also implicated as APCs in the acutely HSV-1-infected mouse cornea [71]. Macrophages infiltrate the TG following HSV-1 infection and aid in eradication of replicating virus in neurons following primary HSV-1 infection and are retained in the TG during latent infection but it remains unclear if they participate in immune responses that prevent HSV-1 reactivation [43, 72]. During acute HSV-1 infection, neurons and Schwann cells in the TG up-regulate the expression of MHCI [73] and it is possible that they also up-regulate MHCII, since IFN-γ that is persistently expressed in the latently infected TG can increase MHCII expression on numerous cell types found in

nervous tissue [74, 75]. As such, it is also possible that neurons and Schwann cells act as APCs to T cells in the TG during HSV-1 latency.

1.3 C57BL/6 MOUSE MODEL OF INFECTION

Several animal models are employed to study HSV-1 infection including guinea pig, rabbit, and mouse models [40, 43, 76-78]. In the proposed studies we employ a C57BL/6 (B6) mouse model of ocular infection and predominantly utilize the HSV-1-KOS strain of virus, a well-characterized strain that was originally isolated from infected human tissue [78, 79]. Ocular infection mimics a common route of infection in humans and like human HSV-1 infection; mice mount an immune response capable of clearing primary infection concurrent with the establishment of latent infection in the TG [39, 78]. Additionally, $CD4^+$ and $CD8^+$ T cell localize around infected neurons in the TG in humans and mice [38, 43] and $CD8^+$ T cells with TCR specificity for the same HSV-1 proteins have been identified in infected mouse and human TGs [38, 40]. Unlike in human infection, spontaneous HSV-1 reactivation rarely occurs in mice [78] and stromal keratitis events are observed following primary infection rather than following a reactivation event, as is most often observed in humans. However, the application of psychological or hormonal stress to latently infected mice can induce *in vivo* HSV-1 reactivation, allowing for monitoring of events occurring during and following viral reactivation [18, 80]. As such, mouse models, including the B6 model, are imperfect yet effective models within which to study immune responses to HSV-1 infection that may parallel human HSV-1 infection.

2.0 SPECIFIC AIMS

Specific Aim 1: Define the HSV-1-specific $CD4^+$ T cell repertoire in the TG during primary and latent infection.

Hypothesis: A majority of $CD4^+$ T cells in the TG during primary and latent HSV-1 infection are HSV-specific and recognize numerous HSV-1 proteins, but the breadth of HSV-1 epitopes recognized by $CD4^+$ T cells during latency is reduced in comparison to primary infection.

Data from our laboratory and others indicates that at least a portion of $CD4^+$ T cells present in the TGs of humans with HSV-1 infection bear TCRs specific for HSV-1 proteins. However, the HSV-1 antigens recognized by $CD4^+$ T cells present in the TG following HSV-1 infection remain undefined. It is also unclear if the entire responding $CD4^+$ T cell population in the TG is HSV-specific and, if so, if this antigen-specific population is proportionately maintained in latent HSV-1 infection and has function in preventing HSV-1 reactivation in the TG.

Specific Aim 2: Identify the cell population(s) $CD4^+$ T cells interact with in the TG during latent HSV-1 infection and determine if these cell populations are $MHCII^+$ and capable of activating $CD4^+$ T cells *ex vivo* in the presence of HSV-1 proteins.

Hypothesis: $CD4^+$ T cells interact with $MHCII^+$ APC populations within the HSV-1-infected TG and these $MHCII^+$ populations are capable of eliciting a cytokine response from $CD4^+$ T cells when HSV-1 antigen is present.

Numerous models of HSV infection have indicated that MHCII$^+$ APC populations can activate CD4$^+$ T cells in an antigen-specific manner in peripheral non-lymphoid tissues. However, it remains unclear if CD4$^+$ T cells are activated by cognate antigen in the TG following HSV-1 infection as MHCII$^+$ populations and CD4$^+$ T cell:cell interactions within the TG have yet to be fully defined.

Specific Aim 3: Determine if CD4$^+$ T cells are the sole source of IL-10 in the latently infected TG and if their presence is required for enhanced CD8$^+$ T cell function *in vivo*.

Hypothesis: CD4$^+$ T cells are a major, though perhaps not sole, source of IL-10 in TGs harboring latent HSV-1 infection and their presence in the TG during latency is required to mediate CD8$^+$ T cell function and modulate HSV-1 reactivation.

Approximately 20% of CD4$^+$ T cell present in the latently infected TG express IL-10. IL-10 has a significant effect on the number and functionality of HSV-1 subdominant epitope-specific CD8$^+$ T cells as well as on HSV-1 reactivation within the latently infected TG. However, it is unclear if the effects of IL-10 on CD8$^+$ T cells are CD4-derived as examination of CD8$^+$ T cell function in the TG upon latent depletion of CD4$^+$ T cells has yet to be examined. It is also unclear if CD4$^+$ T cells are the sole source of IL-10 within the TG during latent HSV-1 infection or if additional immune cell populations secrete IL-10 to regulate CD8$^+$ T cell function.

3.0 GENERAL MATERIALS AND METHODS

3.1 MICE

Female 6–8 week old WT C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6.129S6-Il10^{tmlFlv}/J mice (IL-10-GFP mice) were kindly provided by Dr. Francis Carbone, University of Melbourne and were bred in house following their receipt. B6.Cg-Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$/J (ROSA) mice were purchased from The Jackson Laboratory and were bred in house upon their receipt. All experimental animal procedures were reviewed and approved by the University of Pittsburgh Institutional Animal Care and Use Committee, and the animals were handled in accordance with guidelines established by Institutional Animal Care and Use Committee (IACUC).

3.2 VIRUS AND CORNEAL INFECTIONS

HSV-1-KOS strain was grown in Vero cells and intact virions were isolated on Optiprep gradients according to the manufacturer's instructions (Accurate Chemical and Scientific, Westbury, NY). HSV-1-SV40-Cre (F Strain) virus was generously provided by Bernard Roizman (University of Chicago, Chicago, Illinois) and was grown in Vero cells. Intact virions were isolated in Optiprep gradients as per manufacturer's protocol (Accurate

Chemical and Scientific, Westbury, NY). Mice were anesthetized by i.p. injection of 2.5 mg ketamine hydrochloride and 0.25 mg xylazine (Phoenix Scientific, San Marcos, CA) in 0.25 ml HBSS (BioWhittaker, Walkersville, MD). Mice received bilateral topical infection on scarified corneas with a dose of 1 X 10^5 PFU/eye unless otherwise noted in text.

3.3 TISSUE PREPARATION

Anesthetized mice (2.5mg ketamine hydrochloride and 0.25mg xylazine, i.p.) were injected with 0.3ml heparin (100U/ml) and euthanized by exsanguination. TGs were excised and digested at 37°C in 100ul/TG of DMEM containing 0.02U Liberase (Roche, Indianapolis, IN) for 1 hour before being triturated into single-cell suspensions through the use of a 200ul pipette tip. Spleens were mechanically dissociated through a 40μm nylon screen (Fisher Scientific, Fair Lawn, NJ) and were depleted of red blood cells (RBCs) by a 3-minute incubation with 1X RBC lysis buffer (0.16M NH_4Cl (Fisher) dissolved in distilled water) prior to their use.

3.4 REAGENTS FOR FLOW CYTOMETRY AND FLUORESCENT IMAGING

APC-conjugated H-$2K^b$ tetramers conjugated to the $gB_{498-505}$ peptide provided by the National Institute of Allergy and Infectious Disease Tetramer Core Facility (Emory University Vaccine Center, Atlanta, GA). CD45-PerCP, CD8-PacBlue, CD4-PacBlue, CD8-APC-Cy7, CD4-PE, IFN-γ-PE-Cy7, and IL-10-APC antibodies as well as FcBlock, Cytofix/Cytoperm, and Perm Wash were purchased from BD Biosciences (San Jose, CA).

CD3-APC-eF780, CD4-FITC, and IL-4-AF647 antibodies were purchased from Biolegend (San Diego, CA). IL-17-PE, GrzB-PE, CD4-APC, F4/80-APC, and F4/80-APC-Cy7 antibodies were purchased from eBioscience (San Diego, CA). Live/Dead-Aqua staining kit purchased from Life Technologies (Grand Island, NY). NeuN-AF488 antibody purchased from Millipore (Billerica, MA). Mouse anti-GS primary and goat anti-mouse secondary antibodies purchased from Invitrogen (Grand Island, NY). All extracellular stains generated received Fc Block at a 1:50 dilution. All flow cytometry samples collected on a FACSAria sorting cytometer and analyses conducted in FlowJo Software (Tree Star, Ashland, OR). All statistics generated in Graphpad Prism software (LaJolla, CA) or Microsoft Excel (Redmond, WA).

4.0 ANTIGEN SPECIFICITY OF CD4+ T CELLS IN THE TG AND SPLEEN DURING ACUTE AND LATENT HSV-1 INFECTION

4.1 INTRODUCTION

We have previously defined the HSV-specific CD8+ T cell repertoire in the TG following ocular infection of B6 mice and it has been determined that *all* CD8+ T cells in TG during primary and latent infection are HSV-specific [40, Sarah Bidula, personal communication]. Recently, CD4+ T cells specific for HSV-1 proteins ICP47 & VP16 were identified in HSV-1-infected human TG samples [38] and numerous studies have indicated that a portion of CD4+ T cells responding to HSV-1 infection have antigen specificity for HSV-1 glycoprotein D (gD) [81-84]. Given these observations, we postulate that a majority of CD4+ T cells in the TG during acute infection are HSV-1 specific and that this specificity is also observed in latency but spans fewer HSV-1 epitopes, as is observed following LCMV infection [85]. Here, we describe results from initial experiments investigating the antigen specificity of CD4+ T cells in the TG following HSV-1 infection.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of splenic APCs for CD4+ T cell stimulations

Spleens from naïve B6 mice were harvested and processed into single cell suspensions before $CD4^+$ and $CD8^+$ cells were removed by positive selection using the EasySep (Stem Cell Technologies, Vancouver, BC) CD4 and CD8 positive selection kits as per manufacturer's protocol. Remaining splenic cells were plated in 8ml sterile culture media in a T175 flask before UV-inactivated HSV-1-KOS virus was added at an MOI of 3. After 1 hour, 0.3ug/ml LPS, 0.12μg/ml TNF-α, and 12ug/ml GM-CSF were added to the flask and it was placed back in the 37°C incubator overnight. The next morning, cells were harvested from culture flask by scraping and kept on ice prior to use.

4.2.2 *Ex vivo* $CD4^+$ T cell stimulations

WT female C57BL/6 mice were bilaterally infected with HSV-1-KOS virus. At time points indicated, spleens and TGs were harvested and dispersed into single cell suspensions. In Figures 3, 4 and 6, both TGs from each mouse were pooled and plated in 96-well round-bottom plates (0.5 TG/well) in 100ul culture media (RPMI, 1% penicillin-streptomycin, 1% L-glutamine, 10% fetal bovine serum, 1% HEPES, 1% Sodium pyruvate, 1% Essential Amino Acids). To stimulate $CD4^+$ T cells, one of the following was added to each well: 1) 1ul Leukocyte Activation Cocktail (LAC) containing Golgi Plug (BD), 2) 1 X 10^5 splenic APCs pulsed with whole UV-inactivated HSV-1-KOS and 2ul Golgi Plug (Brefeldin A) (BD), 3) $1X10^5$ non-pulsed splenic APCs, or 4) 100μl of a 100μM gD peptide stock (IPPNWHIPSIQDA, Invitrogen). Cultures were placed in 37°C, 5% CO_2 incubator for 6 hours before cells were washed in 1X PBS and FACS buffer (1X PBS, 1% FBS, 0.1% Sodium Azide) and stained for viability and with a combination of the following antibodies: CD45-PerCP, CD3-e450 or APC-efluor780, CD8-PacBlue or -FITC, and CD4-PE or -FITC. TGs were washed

in FACS buffer and fixed in 100µl Cytofix/Cytoperm (BD) before being washed in 1X Perm Wash (BD) and intracellularly stained for IFN-γ-PE-Cy7 and IL-10-APC. In Figure 3, a separate sample of stimulated cells was stained with IL-4-APC and IL-17-PE. All staining and fixation steps were carried out for 30 minutes at 4°C in the dark and cells were washed and assessed by flow cytometry.

In Figure 5, HSV-1-infected B6 spleens were harvested and processed into singe cell suspensions at time points indicated and $1X10^6$ cells from each sample were plated per well in round-bottomed 96-well plates. Ten-fold serial dilutions of a 1.3mM gD peptide working stock were prepared in sterile culture media and 100ul of each dilution was added to spleen cultures. Cells were cultured at 37°C in 5% CO_2 for 5 hours before cells were washed in 1X PBS and FACS buffer and stained for viability and with anti- CD44-V450 and -CD4-PE-Cy7. Splenic cells were washed in FACS buffer and fixed in 100µl Cytofix/Cytoperm (BD) before being washed in 1X Perm Wash (BD) and intracellularly stained for IFN-γ. All staining and fixation steps were carried out for 30 minutes at 4°C in the dark. Cells were then washed in FACS buffer and assessed by flow cytometry.

4.3 RESULTS

With HSV-1 encoding 84 proteins, each with hundreds of potential peptide epitopes, we began by collaborating with Alessandro Sette & John Sidney (Center for Infectious Disease, Allergy, & Asthma Research, La Jolla Institute for Allergy & Immunology, San Diego, CA) to identify the top 4% of HSV-1 peptides based on those predicted to bind H-2 Ia^b molecules. The IEDB consensus algorithm was used to scan the HSV-1 proteome (GI 9629378) for

peptides predicted to bind class II MHC molecules with high affinity. These peptides were generated for use in *ex vivo* $CD4^+$ T cell stimulation assays designed to identify HSV-specific clones by their cytokine production after incubation with APCs pulsed with the peptides.

Initially, polyclonal stimulation of $CD4^+$ T cells in the TGs of HSV-1-infected B6 mice was performed to establish an appropriate readout for our intracellular cytokine assay. We anticipated that $CD4^+$ T cells present in the TG would predominantly express the T helper cell 1 (Th_1)-associated cytokine IFN-γ, as is commonly observed in viral infection. Indeed, after 6 hours in culture with PMA and ionomycin, 65-75% of $CD4^+$ T cells isolated from the TGs of 8 day post-infection (DPI) mice expressed IFN-γ and a small proportion (15-20%) expressed IL-10 **(Figure 3A-C, G-I)**. Expression of IL-17 and IL-4, associated with Th_{17} and Th_2 lineages, respectively, was not observed (data not shown). Increased expression of IFN-γ or IL-10 expression was not observed in $CD4^+$ T cells from non-infected TG samples after incubation with PMA and ionomycin **(Figure 3, D-F, J-L)**.

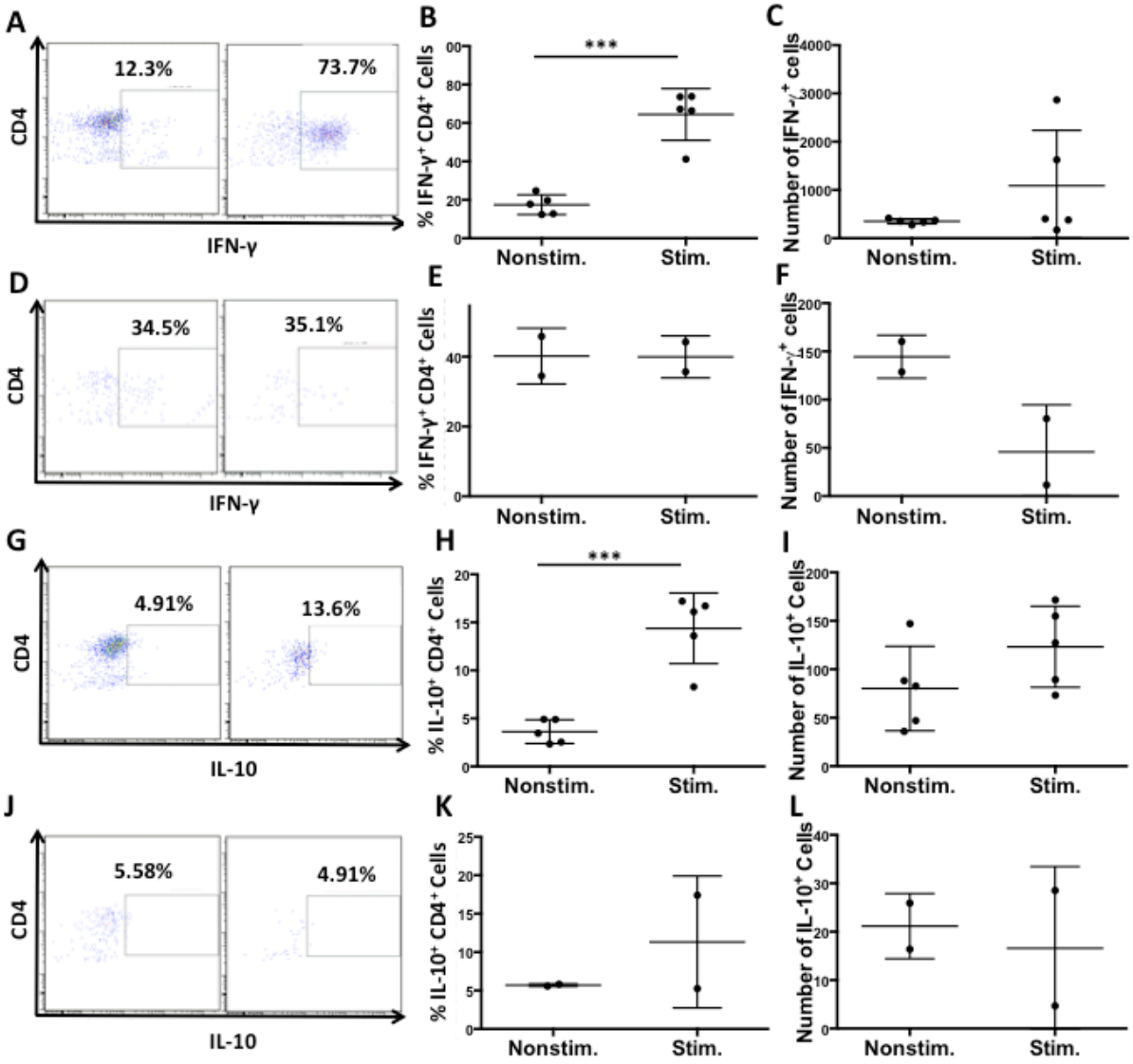

Figure 3. CD4⁺ T cells isolated from C57BL/6 TGs during primary infection express IFN-γ and IL-10 following *ex vivo* culture with PMA and ionomycin.

C57BL/6 mice were bilaterally infected with 1X10^5 PFU of HSV-1-KOS/eye. At 8DPI the TGs from HSV-infected and naïve mice were harvested and processed into single cell suspensions before being cultured for 6 hours in the presence of PMA and ionomycin and Golgi Plug. TG samples were then stained to analyze expression of CD45, CD3, CD4, CD8, IFN-γ and IL-10. (A) shows flow cytometry plots of the IFN-γ expression in CD4⁺ T cells from infected mice that were not stimulated (left) or stimulated with PMA and ionomycin (right). (B) & (C) show the proportion and numbers of CD4⁺ T cells expressing IFN-γ with or without stimulation. (D) shows flow cytometry plots of IFN-γ expression in CD4⁺ T cells from naïve mice with (right) or without (left) PMA and ionomycin stimulation. (E) & (F) show proportion and numbers of naïve CD4⁺ T cells expressing IFN-γ with and without PMA and ionomycin. (G), (H), and (I) show IL-10 expression in CD4⁺ T cells from infected TGs with or without stimulation by PMA and ionomycin and (J), (K), and (L) shows the

same in naïve mice. All flow cytometry plots shown were previously gated on live cells, CD45, and CD3. All bars represent the mean ± SEM of $CD4^+$ T cells. Data are representative of a single experiment (Infected mice, N=5, non-infected mice, N=2). Statistical analyses conducted by utilizing student's t=tests with Welch's correction in GraphPad Prism 6. ****p=0.0007, ***=p=0.0017.

We then examined the overall response of $CD4^+$ T cells isolated from latently infected B6 mice to HSV-1. Pooled TGs were divided and incubated for 6 hours with PMA and ionomycin or with splenic APCs pulsed with UV-inactivated HSV-1-KOS virus. We saw a similar proportional response from $CD4^+$ T cells cultured with PMA and ionomycin at 30DPI as we had observed at 8DPI **(Figure 3 A-C, G-I and Figure 4 A-D)**. $CD4^+$ T cells cultured with splenic APCs pulsed with UV-inactivated HSV-1-KOS also expressed IFN-γ and IL-10 following a 6-hour incubation period **(Figure 4 A-F),** but IFN-γ expression was significantly decreased in comparison to the cells that were stimulated with PMA **(Figure 4 A-B and E).** In contrast, IL-10 expression was moderately increased by HSV-1 stimulation relative to PMA stimulation **(Figure 4 C-D and F)**. $CD4^+$ T cells isolated from the TGs of naïve mice did not express IFN-γ or IL-10 following incubation with PMA and ionomycin or HSV-pulsed splenic APCs (data not shown).

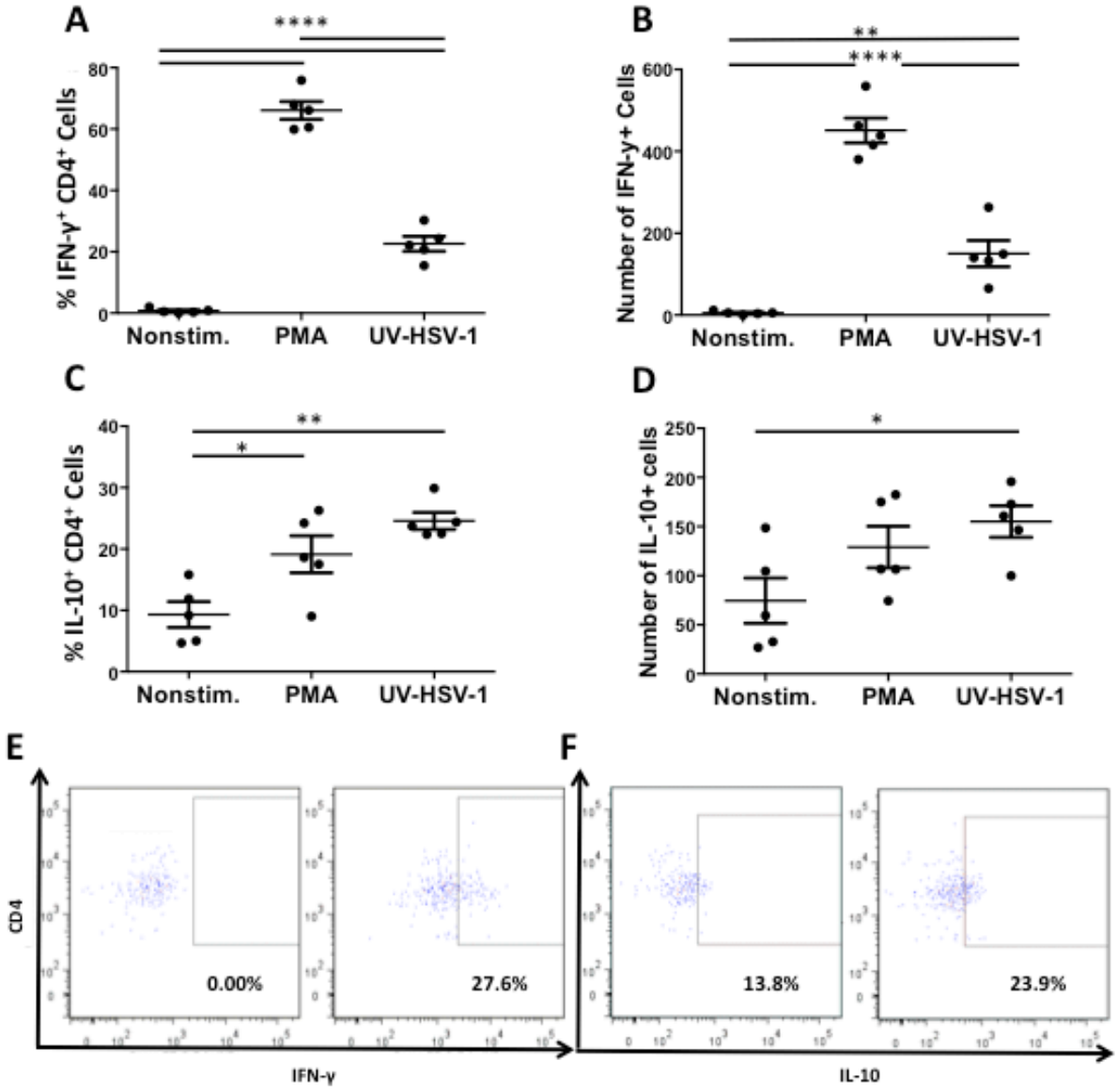

Figure 4. CD4⁺ T cells isolated from C57BL/6 TGs during latent infection express IFN-γ and IL-10 following *ex vivo* culture with splenic APCs pulsed with whole UV-inactivated HSV-1.

C57BL/6 mice were bilaterally infected with $1X10^5$ PFU of HSV-1-KOS/eye. At 30 DPI the TGs from HSV-infected and naïve mice were harvested and processed into single cell suspensions before being cultured for 6 hours in the presence of PMA and ionomycin or splenic APCs pulsed with UV-inactivated HSV-1-KOS. Latently infected TG samples were also cultured with non-pulsed APCs as a negative control. (A) & (B) show the percent and number of CD4⁺ T cells expressing IFN-γ after each culture condition. (C) & (D) show the percent and number of CD4⁺ T cells expressing IL-10 after each culture condition. (E) is a representative flow plot of the UV-HSV-1 stimulation data shown in A with non-stimulated cells on the left and stimulated cells on the right. (F) shows the same but for IL-10 expression. All flow cytometry plots shown were previously gated on live cells, CD45, and CD3. All bars represent the mean ± SEM of CD4⁺ T cells. Data is representative of a single

experiment (n=5). Statistical analyses conducted using a One-Way ANOVA with Bonferroni's Multiple Comparison test in GraphPad Prism 6. ****p<0.0001, **p<0.01, *p=<0.05.

Much evidence has been presented that indicates that HSV-1 glycoprotein D (gD) generates peptide epitopes which are capable of stimulating $CD4^+$ T cell responses following HSV-1 infection [81-84, 86]. Recently, a TCR-transgenic mouse line specific for one epitope of the HSV-1- gD protein was generated by Dr. Andrew Brooks (University of Melbourne, Melbourne, Australia, personal communication). The T cells in these mice recognize a I-Ab-restricted gD epitope that was identified and sequenced by the Brooks laboratory group (unpublished data, personal communication). We obtained this sequence (IPPNWHIPSIQDA) and ordered the corresponding gD peptide from Invitrogen to examine the $CD4^+$ T cell response to this epitope over time. $CD4^+$ T cells isolated from the spleens of B6 mice following HSV-1infection responded to the peptide in a dose-dependent fashion and persisted within the animals for 150+ days **(Figure 5)**.

We also examined the specificity of $CD4^+$ T cells from the TG for their response to this gD peptide at 8DPI. We were unable to detect IFN-γ production in these cells after *ex vivo* culture and a 6 hour incubation with APCs pulsed with a $1X10^{-6}M$ concentration of the peptide **(Figure 6, A-C)**, a concentration that had effectively stimulated splenic $CD4^+$ T cells at 12 and 150 days post-infection **(Figure 5)**.

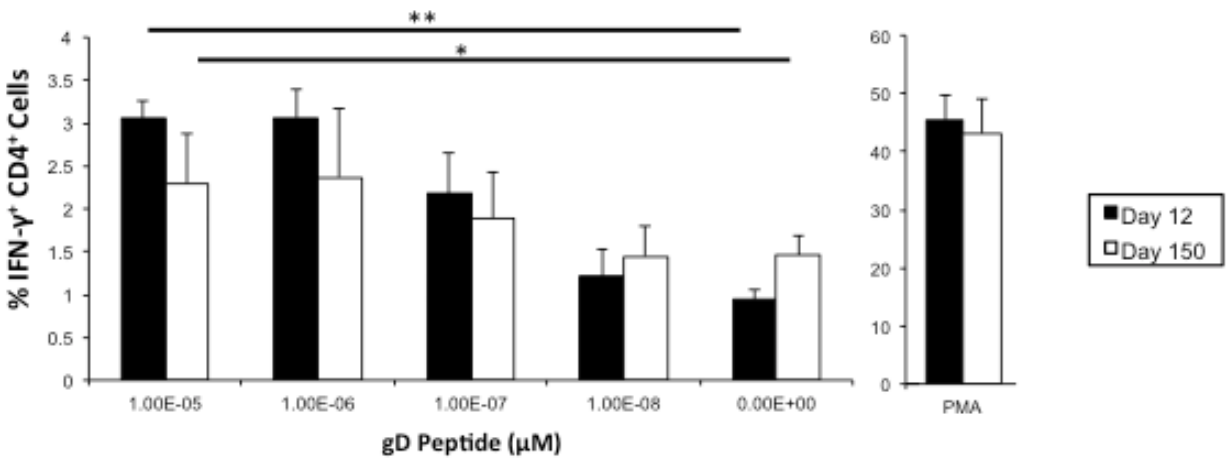

Figure 5. Splenic CD4⁺ T cells have specificity for HSV-1 gD peptide following HSV-1 infection.

C57BL/6 mice were bilaterally infected with 1 X 10^5 PFU of HSV-1-KOS virus/eye. Spleens from infected mice were harvested 12 or 150 DPI and were dispersed into single cell suspensions before being cultured in the presence of serially diluted gD peptide for 5 hours. Cells were then stained for surface expression of CD44 and CD4 and intracellular expression of INF-γ and were analyzed by flow cytometry. The percent of $CD4^+$ T cells expressing IFN-γ at 12 and 150 DPI is shown above. Bars indicate the mean ± SEM. Data are representative of a single experiment (n=3/day12 and 4/day150). Standard student's t tests were conducted in Microsoft Excel to generate statistics**.** *P=0.043, **P<0.01**.** Figure adapted with permissions from data generated by Alexander M. Rowe, Ph.D., Eye and Ear Institute, University of Pittsburgh Medical Center. Pittsburgh, PA.

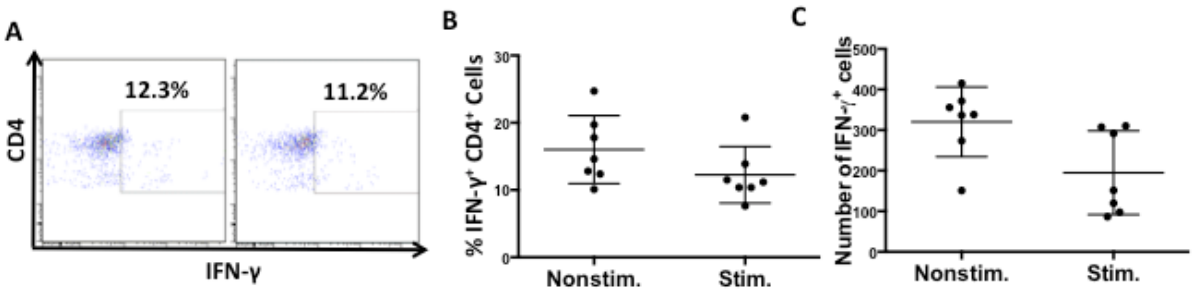

Figure 6. CD4⁺ T cells present in the TG at 8 DPI do not increase IFN-γ production following *ex vivo* culture with splenic APCs and HSV-1 gD peptide.

C57BL/6 mice were bilaterally infected with 1X10^5 PFU of HSV-1-KOS/eye. At 8DPI the TGs from HSV-infected and naïve mice were harvested and processed into single cell suspensions before being cultured for 6 hours in the presence of 100µl gD peptide and 2µl Golgi Plug. TG samples were then stained to analyze expression of CD45, CD3, CD4, CD8, IFN-γ and IL-10. (A) shows flow cytometry plots of the IFN-γ expression in CD4⁺ T cells from infected mice that were not stimulated (left) or stimulated with gD peptide (right). (B) & (C) show the proportion and numbers of CD4⁺ T cells expressing IFN-γ with or without stimulation with gD. All flow cytometry plots shown were previously gated on live cells, CD45, and CD3. All bars represent the mean ± SEM of CD4⁺ T cells. Data is representative of a single experiment (N=7).

4.4 DISCUSSION AND FUTURE DIRECTIONS

A significant portion of the CD4⁺ T cells present in the TG during latent infection are activated in response to HSV-1 *ex vivo* **(Figure 4)**, indicating that these infiltrating CD4⁺ T cells have TCR specificity for HSV-1 peptides. However, we anticipated a larger proportion of CD4⁺ T cells isolated from latently infected TGs would be responsive to HSV-1 *ex vivo*, as is observed for CD8⁺ T cells [40]. As such, we conducted numerous experiments to see if a greater response could be achieved following the *ex vivo* stimulation. We increased the effector APC to CD4⁺ T cell target ratio, increased the multiplicity of infection (MOI) of the UV-inactivated HSV-1-KOS virus in the APC cultures, extended the time of culture from 6 to 10 hours, and generated BMDCs from naive mice to serve as stimulating APCs in place of

splenic APCs. The cytokine expression within the $CD4^+$ T cells of IFN-γ-Thy1.1 and IL-10-GFP reporter mice was also examined following infection to eliminate the possibility that our fluorescent antibodies were of low specificity or affinity. However, we were unable to get a greater proportional response than is presented here.

A future experiment examining the levels of markers for exhaustion, such as PD-1 and Tim-3, in addition to cytokine expression in $CD4^+$ T cells from latently infected TGs following *ex* vivo stimulation should also be conducted. It may show that the non-responsive $CD4^+$ T cells are exhausted, leaving open the possibility that the cells are HSV-1-specific but are incapable of eliciting effector functions. Future troubleshooting should also include extending the length of the *ex vivo* culture time to 16-24 hours rather than 6 or 10. $CD4^+$ T cells have been shown to require a greater period of exposure to antigen to become fully activated than is observed for $CD8^+$ T cells and extending the length of stimulation may increase the proportion of IFN-γ^+ and IL-10^+ $CD4^+$ T cells detected. It may also be of benefit to compare the response of $CD4^+$ T cells to whole UV-inactivated virus to that of a Vero cell lysate harvested 10 hours post-infection. The UV-inactivated viruses used are nearly, but not completely, inactivated and thus may be capable of infecting and consecutively retarding efficient antigen presentation by splenic APCs or BMDCs to $CD4^+$ T cells.

It is also feasible that $CD4^+$ T cells in the TG that are not activated in our *ex vivo* cultures may not have TCR specificity for HSV-1 peptides. An experiment examining the $CD4^+$ T cytokine response to whole UV-inactivated HSV-1 in comparison to a pool of all of the predicted MHCII-binding HSV-1 peptides may prove telling. If the magnitude of the *ex vivo* response of $CD4^+$ T cells to the conglomerate peptides is equivalent to that of $CD4^+$ T cells

to whole virus, it may indicate that the remaining $CD4^+$ T cell population in the TG is not specifically responding to HSV-1.

While we were able to generate a response to the HSV-1 gD peptide from splenic $CD4^+$ T cells at 12 and 150DPI, we did not observe cytokine secretion from $CD4^+$ T cells isolated from TGs in response to gD peptide at 8DPI **(Figures 5-6)**. An experiment conducted in our laboratory indicates that the highest numbers of $CD4^+$ T cells can be found in the TG at 11DPI. The lack of response by $CD4^+$ T cells to the HSV-1 gD peptide at 8DPI may be the result of examining the CD4 response at a time point too early after infection when all of the HSV-1-specific $CD4^+$ T cell clones have not yet migrated to the TG. Culturing the TGs with gD peptide and a larger APC population than that native to the TG or examining the gD response of $CD4^+$ T cells in the TG at later time points post-infection will likely indicate if these $CD4^+$ T cells have TCR-specificity for this gD epitope.

5.0 CD4+ T CELL INTERACTIONS IN THE LATENTLY INFECTED TG

5.1 INTRODUCTION

It remains unclear if CD4+ T cells found in the latently infected TG are specific for HSV-1 antigen and, if so, if they are exposed to antigen within this tissue. Studies conducted by the Verjans laboratory indicate that Satellite Glial Cells (SGCs) within the human TG bear an APC phenotype, showing expression of numerous APC markers including CD11c, CD80 and 86, and MHCII [87]. vanVelzen and Verjans also reported that CD4+ T cells in HSV-1-infected human TGs are found surrounding neurons in clusters, display antigen-specificity for a broad range of HSV-1 proteins, and show evidence of recent activation [38] indicating that CD4+ T cells are likely being activated in the local tissue and not in the periphery prior to migration to the TG. It has also been well demonstrated that DC populations are capable of eliciting robust antigen-specific responses from both CD4 and CD8+ T cells present in peripheral tissues following viral infection, including HSV-1 [63, 66-68]. Given these findings, it appears that both hematopoietic and non-hematopoietic cell populations may be capable of serving as APCs to activate CD4+ T cells within the TG.

Data providing direct evidence of CD4+ T cell activation in the TG has yet to be published and HSV-1 proteins are rarely detected in the TG during studies examining latent HSV-1 infection, calling into question the availability of antigen for APCs to acquire and present. However, HSV-1 lytic gene transcripts have been identified in the neurons of

infected human TGs and neuronal cells positive for these transcripts have increased numbers of $CD8^+$ T cells surrounding them [36]. We have shown that nearly all $CD8^+$ T cells in the TG during latent HSV-1 infection are antigen specific. And that non HSV-specific $CD8^+$ T cells are lost from the latently infected TG over time. This implies that viral proteins persistently stimulate the T cells within the latently infected TG. Indeed, it is possible that HSV-1 proteins are produced but are produced at levels below detection and/or are taken up and processed too rapidly to accumulate to detectable levels. We postulate that $CD4^+$ T cells are activated in an HSV-specific manner within the latently infected TG and we wish to identify the $MHCII^+$ APC population(s) that stimulates $CD4^+$ T cells in this tissue. Here, we describe the results of 2 experiments examining the interactions of $CD4^+$ T cells in the latently infected TG.

5.2 MATERIALS AND METHODS

C57BL/6 mice were bilaterally infected with 1 X 10^5 PFU HSV-1-KOS/cornea. B6.Cg-Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$/J (ROSA) mice were infected bilaterally with 3X10^5 PFU HSV-1-SV40-Cre virus/ cornea. TGs were harvested from mice at time points indicated and were imaged non-fixed before a 24-hour room-temperature fixation in 4% PFA (Figure 8) or were placed in 4% PFA immediately without prior imaging (Figure 7). TGs were treated with sodium borohydrate ($NaBH_4$) to reduce autofluorescence and permeabilized in 0.3% TritonX-100 in 1X PBS containing 2% BSA. Both incubations were carried out for 30 min. at room temp. ROSA TGs were then stained with anti-NeuN-AF488 and anti-CD4-APC (Figure 8) and B6 TGs were stained with anti-NeuN-AF488, anti-CD4-PE, anti-F4/80-APC, and

primary mouse anti-glutamine synthetase (GS) antibody (Figure 7). All stains prepared in blocking buffer (0.3% TritionX-100 and 0.1% Tween-20 in 1X PBS) and staining carried out overnight in the dark at 4°C. TGs shown in figure 7 were also stained for 2 hours in the dark in a secondary goat anti-mouse-PacBlue antibody. TGs were whole mounted on glass slides with coverslips and images collected on an Olympus Fluoview 1000X confocal microscope. Images captured with a 20X. 0.85NA (8, A & B) or 40X, 1.3NA (8, C & D) oil immersion objective. All image reconstructions created using Olympus Fluoview and Fiji software. Images were adjusted for display.

5.3 RESULTS

In a preliminary experiment C57BL/6 mice were bilaterally infected with $1X10^5$ PFU of HSV-1-KOS virus/cornea. At 30 DPI TGs were harvested, whole mounted, and antibody-labeled for CD4, NeuN, glutamine synthetase (GS), and F4/80 and were then imaged by confocal microscopy. We observed $CD4^+$ T cell colocalization with $F4/80^+$ cells and in close apposition to GS^+ and $NeuN^+$ cells **(Figure 7)**, indicating that there are potentially direct interactions occurring between $CD4^+$ cells and macrophages, Satellite Glial Cells (SGCs), and/or neurons within the TG.

To delineate whether or not $CD4^+$ T cells present in the latently infected TG interact specifically with HSV-1-infected neurons, we utilized the B6.Cg-Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$/J strain of mice whose cells express the fluorescent TdTomato protein in the presence of Cre, a DNA recombinase [88]. These mice were infected with an HSV-1 virus engineered to express Cre protein from an SV40 promoter. Expression of Cre in neurons in

the ROSA mouse results in expression of the fluorescent TdTomato protein within infected neurons and these cells will glow red in comparison to non-infected neuronal cells. As such, we believe we can utilize this system to examine $CD4^+$ T cell interactions in the intact TG and determine if these cells are specifically interacting with HSV-infected neurons.

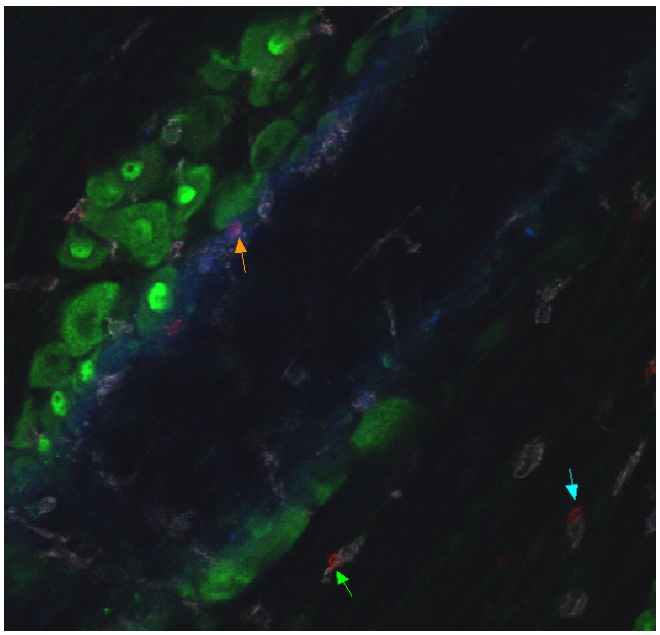

Figure 7. $CD4^+$ T cells are in close apposition to $F4/80^+$ cells and neurons in latent HSV-1 infection in WT B6 mice.

C57BL/6 mice were bilaterally infected with $1X10^5$ PFU HSV-1-KOS virus. At 30 DPI TGs were harvested and fixed in 1% PFA before being stained for NeuN (green), CD4 (red), F4/80 (gray), and glutamine synthetase (GS) (blue). TGs were whole mounted and imaged by confocal microscopy (10X). Blue and green arrows indicate $CD4^+$ cells in close proximity to $F4/80^+$ cells and orange arrow indicates $CD4^+$ cell in close apposition to a neuron or satellite cell, all of which are potentially $MHCII^+$ APC populations capable of stimulating $CD4^+$ cells within the TG.

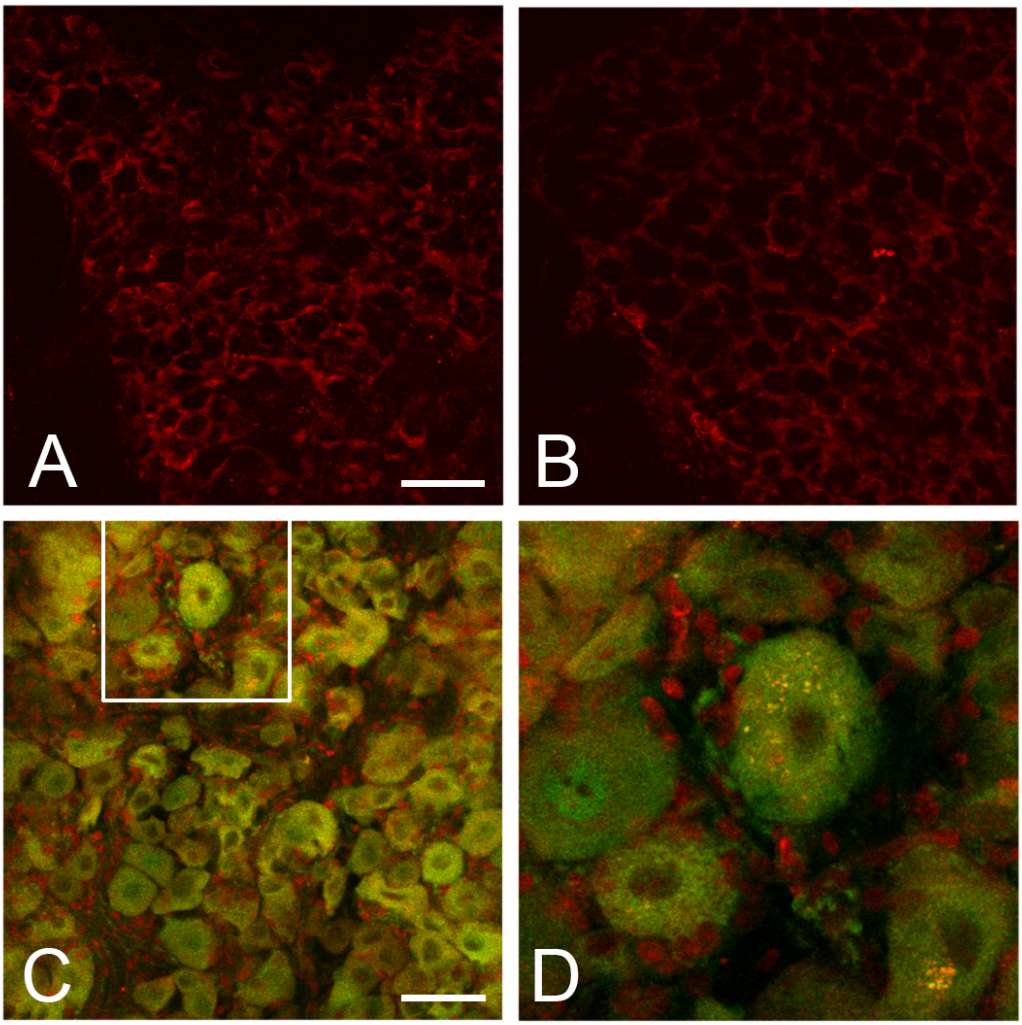

Figure 8. CD4+ T cells are present in high numbers and apposed to neurons during acute HSV-1 infection in ROSA-TdTomato mice.

B6.Cg-Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$/J mice were bilaterally infected with 3 X 10^5 PFU of HSV-1-SV40-Cre virus. At 10 DPI, naïve (A) or latently infected (B) TGs were harvested and whole mounted. TdTomato expression was assessed by confocal microscopy (20x, Scale bar = 100um) and clearly demonstrates a lack of expression in infected TGs in comparison to non-infected samples. HSV-1-infected TGs were then fixed and stained (C, with a close-up image shown in D) with anti-NeuN-488 (green) and anti-CD4-APC (red) before imaging (40x, Scale bar = 50um). CD4+ T cells are shown surrounding neurons, indicating potential for TCR:peptide:MHCII interactions and CD4+ T cell activation.

Ten days following HSV-1-Cre infection, ROSA TGs were harvested and whole mounted live for confocal imaging. We noticed a high level of background fluorescence in both infected and non-infected TG samples **(Figure 8, A & B)**. Given these high levels of background fluorescence, we were unable to clearly identify HSV-infected versus non-infected neuronal cells. We chose to fix and stain these TGs to determine $CD4^+$ T cell presence and localization within the TG. Whole TGs were stained with anti-CD4 and anti-NeuN prior to being imaged by confocal microscopy. A large $CD4^+$ T cell infiltrate was present in the TGs of HSV-1-Cre infected ROSA mice at 10DPI and a majority of these $CD4^+$ T cells were in close apposition to neurons **(Figure 8, C & D)**.

5.4 DISCUSSION AND FUTURE DIRECTIONS

We were unable to identify infected neurons based on TdTomato expression in our ROSA mice infected with HSV-1-SV40-Cre virus, even when using a dose of virus 3 times greater than that typically given of our laboratory HSV-1-KOS strain. This prevented us from assessing the specificity of $CD4^+$ T cell associations with infected cells versus interactions with non-infected cells in the TG at 10DPI **(Figure 8, A & B)**. However, additional HSV-1-Cre viruses, generously provided by Dr. Stacey Efstathiou (University of Cambridge, Cambridge, UK), will be examined in ROSA mice in future experiments. The HSV-1-Cre viruses provided by Dr. Efstathiou have been well established as effective tools with which to study HSV-1-infection in mouse models of neuronal infection [89-91] and may allow us to more readily differentiate between infected and non-infected neuronal cells within the TG.

While we could not identify infected neurons in the TG at 10 DPI with certainty, we did observe a significant $CD4^+$ T cell infiltrate within the TG and a majority of these cells were closely apposed to neurons **(Figure 8, C & D)**, indicating potential direct interactions with these cells. The numbers of $CD4^+$ T cells in the TG during primary infection were much greater than that observed in latent infection, consistent with observations made of $CD8^+$ cells in our previous studies [43, 92-94]. As $CD4^+$ T cells are in close apposition to neuronal cells within the TG during both acute and latent infection **(Figures 7 & 8)**, neurons or the satellite cells associated with them are of key interest to us in our studies of antigen presentation to $CD4^+$ T cells in the TG. The association of $CD4^+$ T cells with neurons following HSV-1 infection is in agreement with observations made in human tissues that indicate that HSV-1-specific $CD4^+$ T cells cluster around neurons, particularly those positive for HSV-1 transcripts [38], bolstering our hypothesis that $CD4^+$ T cells in the latently infected TG are an HSV-specific population that is exposed to antigen in this tissue. However, additional studies are needed to examine the MHCII expression within neurons and SGCs in the TG. Additionally, *ex vivo* assessment of their ability to activate $CD4^+$ T cells isolated from the TGs of HSV-1-infected mice must be conducted to determine if these cell populations are in fact capable of activating $CD4^+$ T cells in the presence of HSV-1.

In addition to neurons, $CD4^+$ T cells were also in close apposition to cells expressing F4/80 within the TG during latent HSV-1 infection **(Figure 7)**, which may be tissue-resident DCs or macrophages that can act as APCs to $CD4^+$ T cells during latency. We did not assess $F4/80^+$ cell:$CD4^+$ T cell interactions in primary infection so it is unclear if this same pattern of localization exists early in HSV-1 infection. In future studies we will attempt to better define this $F4/80^+$ population by staining for additional markers of

myeloid populations, such as Ly6C, CD11b, and CD11c [95]. This will help us determine if these cells are a tissue-resident DC population or another myeloid population such as macrophages. If we observe that the cells opposed to $CD4^+$ T cells in the TG are $CD11b^+$ and $F4/80^+$, but CD11c and Ly6C negative, we can be more confident that these cells are tissue-resident macrophages. Tissue-resident DCs have been observed to stimulate $CD4^+$ T cells in the skin following HSV-1 infection [96] and this may also occur in the TG. If expression of Ly6C is observed, the cells are likely a macrophage population, which may also be capable of presenting antigen to $CD4^+$ T cells. We will also examine MHCII expression in these cells and assess the ability of this myeloid population to activate $CD4^+$ T cells isolated from HSV-1-infected TGs *ex vivo*.

6.0 THE ROLE OF IL-10 IN THE TG DURING LATENT HSV-1 INFECTION: SOURCES OF IL-10 IN THE LATENTLY INFECTED TG AND THE EFFECT OF CD4 DEPLETION ON CD8 FUNCTION AND VIRAL LATENCY

6.1 INTRODUCTION

Recent studies in our laboratory have shown that nearly 20% of $CD4^+$ T cells in the TG during latent HSV-1 infection are expressing the anti-inflammatory cytokine IL-10 and that blockade of IL-10 receptor results in an increase in the functionality of subdominant epitope-specific $CD8^+$ T cells and a reduced rate of HSV-1 reactivation [45]. IL-10 is known to have suppressive functions in mediating the immune response to numerous insults, including viral infections. We hypothesize that $CD4^+$ T cells mediate $CD8^+$ T cell functions within the latently infected TG thus, in part, mediating HSV-1 latency and reactivation events. We postulate that IL-10 may be a critical chemical factor secreted by $CD4^+$ T cells to mediate $CD8^+$ T cell functions. However, we cannot discount the possibility that additional immune cell populations may also produce IL-10 to mediate HSV-1 latency in the TG. Here, we examine $CD8^+$ T cell function following $CD4^+$ T cell depletion during latency and also examine the TG for additional sources of IL-10 in hopes of gaining a better understanding of the role of IL-10 and $CD4^+$ T cells in latent HSV-1 infection.

6.2 MATERIALS AND METHODS

6.2.1 *In vivo* CD4$^+$ T cell depletions

Latently infected C57BL/6 (Figure 9) or B6.129S6-Il10^{tmlFlv}/J (Figures 10 & 11) mice were administered 150µg in 100µl of anti-CD4 antibody (BioXCell, West Lebanon, NH) or 100µl 1X PBS by i.p. injection at 30, 35, 40, and 45 days post-infection. TGs were harvested at 46 DPI and were processed into single cell suspensions before being stained for 30 minutes at room temperature with the following: anti-CD4-PacBlue, anti-CD8-APC-Cy7, anti-CD45-PerCP, and gB tetramer. Samples shown in Figure 11 were also stained with anti-PD-1. Cells were washed in FACS buffer and fixed in 100µl Cytofix/Cytoperm (BD) before being washed in 1X Perm Wash (BD) and stained for intracellular GrzB. Fixation and intracellular staining steps were carried out in the dark at 4°C for 30 minutes. Cells were then assessed by flow cytometry.

6.2.2 Assessment of IL-10 production in the TG following HSV-1 infection

TGs from B6.129S6-Il10^{tmlFlv}/J mice were harvested and processed into single cell suspensions at time points indicated. In Figure 12, TGs from each mouse were pooled and divided in 4 (1/2 TG/sample) before being stained with: anti- CD45-PerCP, CD4-PE, and F4/80-PE-Cy7 in FACS buffer. In Figure 13, whole TGs were stained with: anti- CD45-BV510, CD4-PacBlue, and F4/80-PE-Cy7 in FACS buffer. All samples stained for 30 minutes at 4°C in the dark. Following staining all samples were fixed in 100µl Cytofix/Cytoperm (BD) at 4° for 20 min. before being washed in FACS buffer and analyzed by flow cytometry.

6.3 RESULTS

To examine the functions of IL-10 in latent HSV-1 infection, we first examined $CD8^+$ T cell function upon $CD4^+$ T cell depletion during latent HSV-1 infection. We observe exclusive expression of IL-10 by $CD4^+$ T cells in latent TGs and that blockade of IL-10R increased the number and function of non-gB-specific $CD8^+$ T cells [45]. As such, we hypothesized that depletion of $CD4^+$ T cells from the TG during latent infection would result in the same increase in number and function of non-gB-specific $CD8^+$ T cells seen with IL-10R blockade. In a pilot study, $CD4^+$ T cells were depleted from latently infected B6 mice and epitope-specific $CD8^+$ T cell function was assessed **(Figure 9)**. Intraperitoneal injection of 150μg of anti-CD4 neutralizing antibody every five days over the course of 20 days resulted in a significant reduction in CD4 numbers, both in the TG **(Figure 9, A-C)** and systemically (data not shown). However, the depletion was incomplete and a significant population of $CD4^+$ T cells still remained in the ganglia following depletion **(Figure 9, A-C)**. Examination of the $CD8^+$ population following $CD4^+$ T cell depletion showed a negligible decrease in $CD8^+$ T cell numbers **(Figure 9D)** and that the proportion and number of gB-specific and non gB-specific cells within the $CD8^+$ population were not altered **(Figure 9, E-F)**. Additionally, expression of GrzB and PD-L1 remained unchanged in both populations **(Figure 9, G-J)**. It is to be noted that the overall numbers of both $CD4^+$ and $CD8^+$ T cells in infected and non-depleted mice in this experiment were significantly lower than is typically seen in the C57BL/6 model of HSV-1 infection in our laboratory.

Our preliminary study showed no alterations to $CD8^+$ T cell upon $CD4^+$ T cell depletion. However, the depletion was incomplete **(Figure 9, A-C)** so we could not determine if these were valid observations of the function of $CD8^+$ T cells in the absence of a robust $CD4^+$ T

cell population or if they were the result of an incomplete depletion of $CD4^+$ T cells. As such, we repeated the experiment using B6.129S6-Il10^{tmlFlv}/J mice. These mice express a bicistronic message from the IL-10 promoter that encodes the IL-10 cytokine as well as green fluorescent protein (GFP). Expression of GFP from the mRNA occurs via an internal ribosome entry site (IRES) that is 3' to the IL-10 protein-coding region while the IL-10 mRNA is translated following traditional 5' cap-binding by cellular ribosomes. Thus, these mice can be used to monitor IL-10 expression via GFP with minimal disruption to native IL-10 protein expression. Female mice were infected and depleted of $CD4^+$ cells as before, and $CD8^+$ T cell function in the TG was assessed **(Figure 10)**. Initial $CD4^+$ and $CD8^+$ T cell numbers were higher than in our pilot study **(Figure 10, A-D)** and we saw a proportionately greater reduction in $CD4^+$ T cells than was observed in our first experiment **(Figure 10B)**. While a smaller percentage of $CD4^+$ T cells was observed, there was still a greater number of $CD4^+$ T cells remaining in the TG following depletion in our second experiment than were remaining in our pilot depletion experiment **(compare Figure 10C to Figure 9C).**

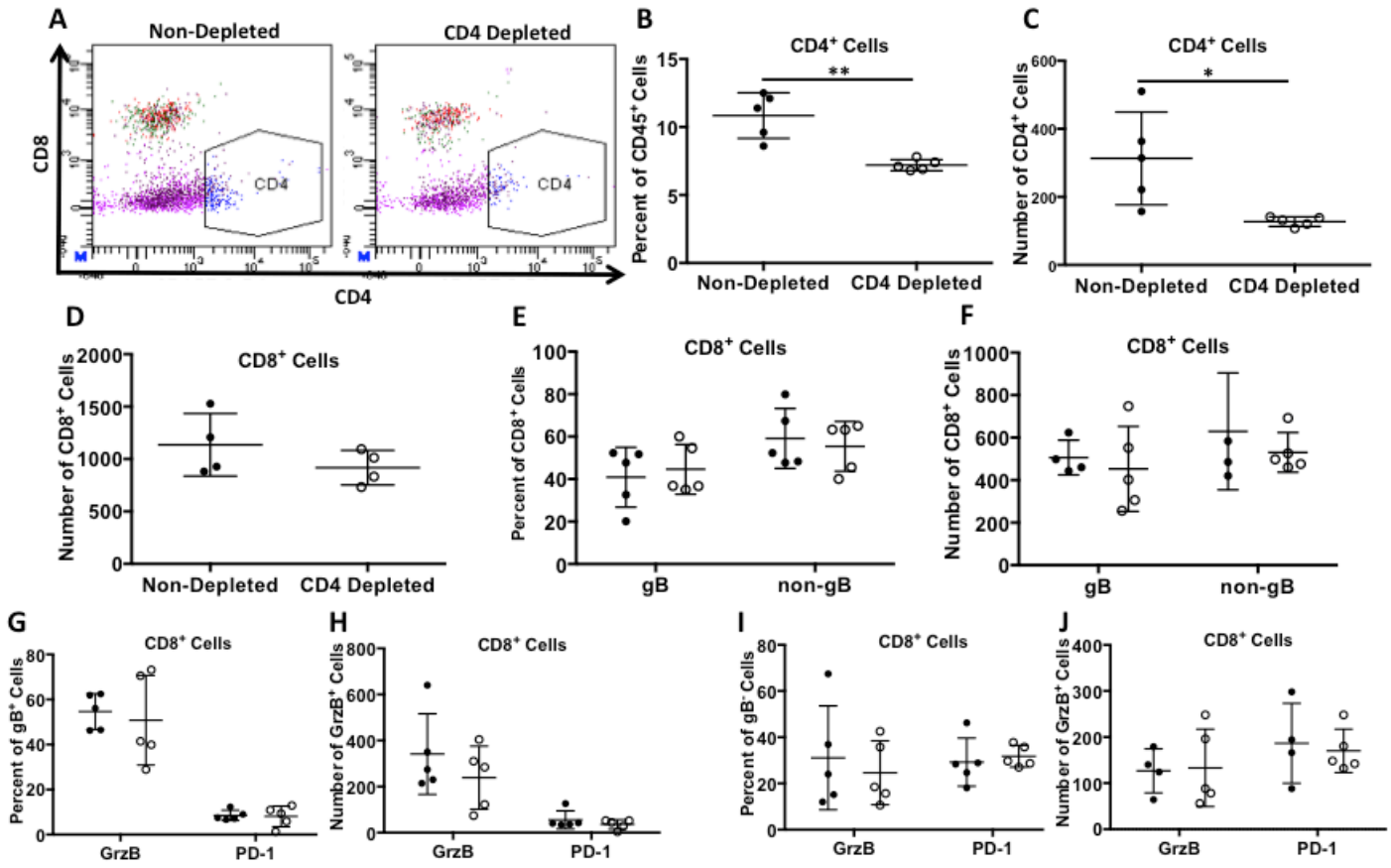

Figure 9. Partial depletion of CD4⁺ T cells from the TG during HSV-1 latency in B6 mice does not result in an increase in epitope-specific CD8⁺ T cell numbers or functionality.

B6.129S6-Il10^{tmvFlv}/J mice were bilaterally infected with 1X10^5 PFU HSV-1-KOS. At 30, 35, 40, and 45DPI, 150µg anti-CD4 antibody or 100µl PBS was administered by intraperitoneal injection. TGs were harvested at 46DPI and processed into single-cell suspensions before being stained with anti-CD45, CD8, CD4, gB, PD-1, and GrzB antibodies and CD8 function assessed by flow cytometry. (A) shows representative flow plots of CD4⁺ and CD8⁺ T cells isolated from B6 TGs that were not depleted (left) or were depleted (right) of CD4⁺ T cells. (B) shows a graphical representation of the data represented in (A). The number of CD4⁺ and CD8⁺ T cells found in the TGs of B6 mice that have or have not been depleted of CD4⁺ cells are shown in (C) and (D). The proportion (E) and number (F) of gB-specific and non gB-specific cells within the CD8⁺ T population with (open circles) or without (filled circles) CD4⁺ T cell depletion are indicated. The proportion and numbers of gB+ (G-H) and gB- (I-J) CD8⁺ T cells expressing GrzB and PD-1 with (open circles) or without (filled circles) CD4 depletion are shown in (G-J). All flow cytometry plots shown were previously gated CD45. All bars represent the mean ± SEM of cell type indicated. Data is representative of a single experiment (N=5/treatment group). Statistical analyses conducted using a student's t-test with Welch's correction in GraphPad Prism 6. **p=0.0069, *p=0.0376.

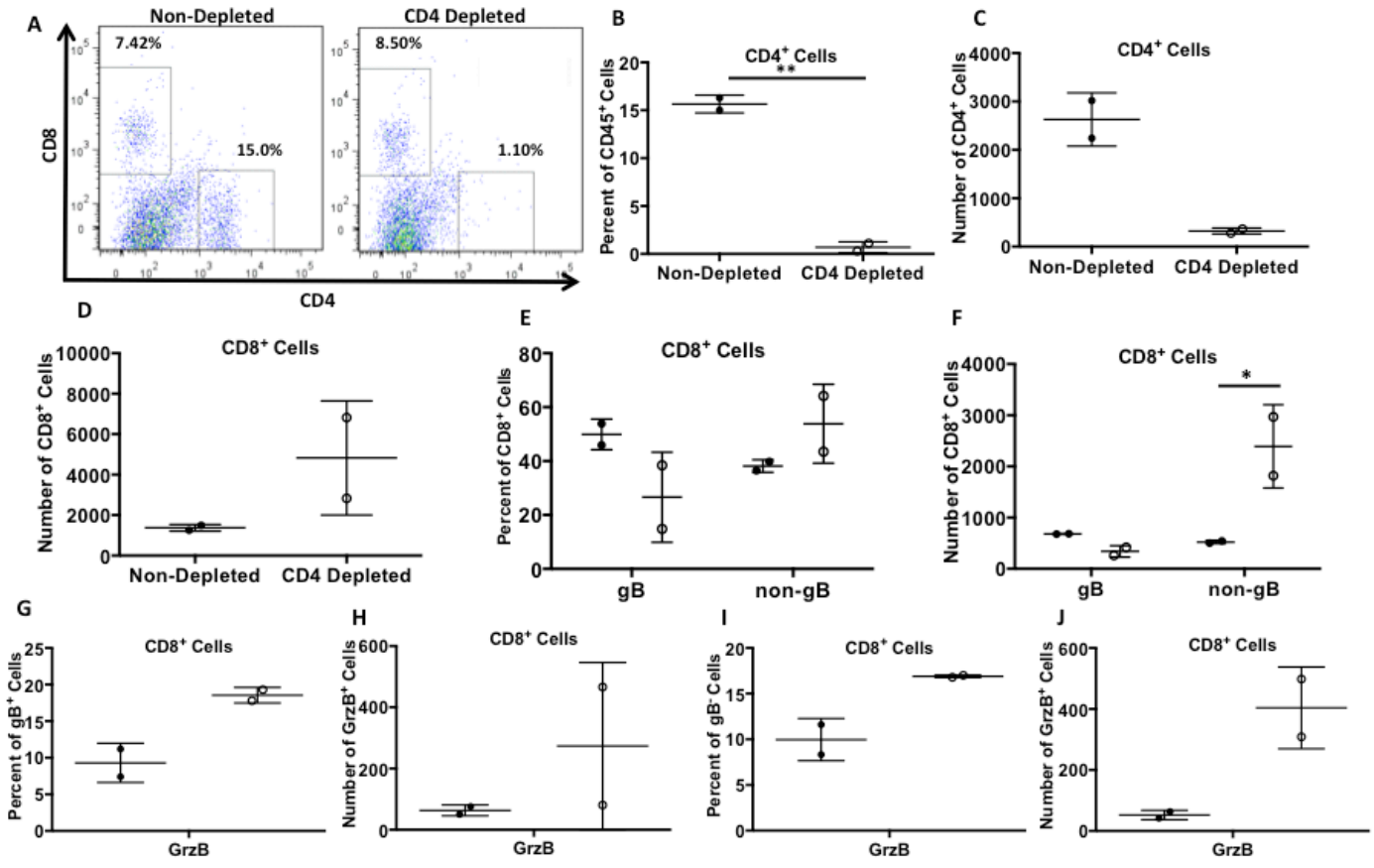

Figure 10. Partial depletion of CD4+ T cells from the TGs of IL-10-GFP mice during HSV-1 latency results in a significant increase in non-gB-specific CD8+ T cell numbers and an increase in GrzB expression.

B6.129S6-Il10^{tmvFlv}/J mice were bilaterally infected with 1X10^5 PFU HSV-1-KOS virus. At 30, 35, 40, and 45DPI, 150µg anti-CD4 antibody or 100µl PBS was administered by intraperitoneal injection. TGs were harvested at 46DPI and processed into single-cell suspensions before being stained with anti- CD45, CD8, CD4, gB, and GrzB antibodies and CD8 function assessed by flow cytometry. (A) shows representative flow plots of CD4+ and CD8+ T cells isolated from B6 TGs that were not depleted (left) or were depleted (right) of CD4+ T cells. (B) shows a graphical representation of the data represented in (A). The number of CD4+ and CD8+ T cells found in the TGs of B6 mice that have or have not been depleted of CD4+ cells are shown in (C) and (D). The proportion (E) and number (F) of gB-specific and non gB-specific cells within the CD8+ T population with (open circles) or without (filled circles) CD4+ T cell depletion are indicated. The proportion and numbers of gB+ (G-H) and gB- (I-J) CD8+ T cells expressing GrzB with (open circles) or without (filled circles) CD4 depletion is shown in (G-J). All flow cytometry plots shown were previously gated CD45. All bars represent the mean ± SEM of cell type indicated. Data is representative of a single experiment (N=2/treatment group). Statistical analyses created using GraphPad Prism 6. Statistic in (B) was generated by conducting a student's t-test with Welch's correction, **p=0.0058. Statistic in (F) was generated by conducting a Two-way ANOVA with multiple comparisons, *p<0.05.

In our second $CD4^+$ T cell depletion experiment we observed a slight increase in overall $CD8^+$ T cell numbers and a significant increase in the number of subdominant epitope-specific $CD8^+$ T cells in the TG that had not been seen in our pilot experiment **(compare Figure 10, D-F to Figure 9, D-F)**. Additionally, we observed a trend of increased GrzB expression in both gB-specific and non gB-specific $CD8^+$ T cells following CD4 depletion that was also not observed in our pilot study **(compare Figure 10, G-J to Figure 9, G-J).** We also attempted to examine HSV-1 reactivation in TGs depleted or not depleted of $CD4^+$ T cells *ex vivo*, but results from this study were inconclusive (data not shown). As such, it remains unclear if this increase in non-gB $CD8^+$ T cells and GrzB expression translates to a reduced frequency of HSV-1 reactivation.

To determine if remaining $CD4^+$ T cells had an altered IL-10 expression phenotype, we examined the IL-10-GFP MFI within these cells and compared it to that in $CD4^+$ T cells from non-depleted mice. We found that there was roughly the same number of $CD4^+$ T cells expressing IL-10 in TGs depleted of $CD4^+$ cells as there were in TGs that had not been depleted of $CD4^+$ cells **(Figure 11B)**. All $CD4^+$ T cells remaining in the TGs following depletion were IL-10^+, whereas only 10-15% of the $CD4^+$ T cells harvested from the TGs of mice not depleted of $CD4^+$ T cells were IL-10^+. **(Figure 11, A & C)**. However, the IL-10-GFP mean fluorescence intensity (MFI) was greatly reduced in the IL-10^+ $CD4^+$ T cell population in the TGs of CD4-depleted mice in comparison to IL-10^+ $CD4^+$ T cells in non-depleted mice **(Figure 11D)**. This reduction in MFI was not statistically significant, but the pattern of reduced IL-10 levels within the $CD4^+$ cells was apparent.

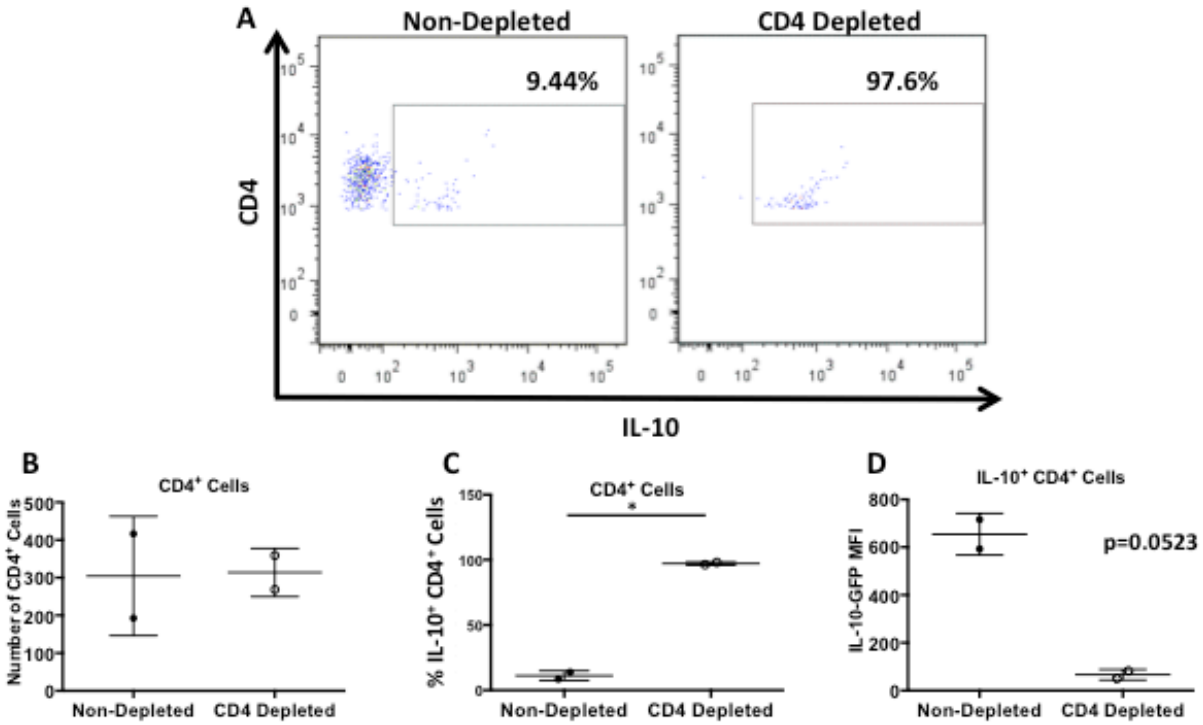

Figure 11. CD4⁺ T cells remaining in the TG following *in vivo* CD4⁺ T cell depletion are IL-10⁺, but express less IL-10/cell than CD4⁺ T cells in TGs from non-depleted mice.

B6.129S6-Il10^{tmvFlv}/J mice were bilaterally infected with 1X10^5 PFU HSV-1-KOS virus. At 30, 35, 40, and 45DPI, 150µg anti-CD4 antibody or 100µl PBS was administered by intraperitoneal injection. TGs were harvested at 46DPI and processed into single-cell suspensions before being stained with anti-CD45 and anti-CD4 and IL-10-GFP expression assessed by flow cytometry. (A) shows representative flow plots of IL-10 expression in CD4⁺ T cells isolated from B6.129S6-Il10^{tmvFlv}/J TGs that were not depleted (left) or were depleted (right) of CD4⁺ T cells. The number of CD4⁺ T cells expressing IL-10 in the TGs of B6.129S6-Il10^{tmvFlv}/J mice that have or have not been depleted of CD4⁺ cells is shown in (B). (C) shows a graphical representation of the data represented in (A). (D) is a representative graph of the IL-10-GFP MFI in CD4⁺ T cells isolated from non-depleted or CD4⁺ T cell depleted IL-10-GFP reporter mice. All flow cytometry plots shown were previously gated CD45. All bars represent the mean ± SEM of cell type indicated. Data is representative of a single experiment (N=2/treatment group). Statistical analyses created using GraphPad Prism 6. Statistic in (C) generated by conducting a student's t-test with Welch's correction, *p=0.0103.

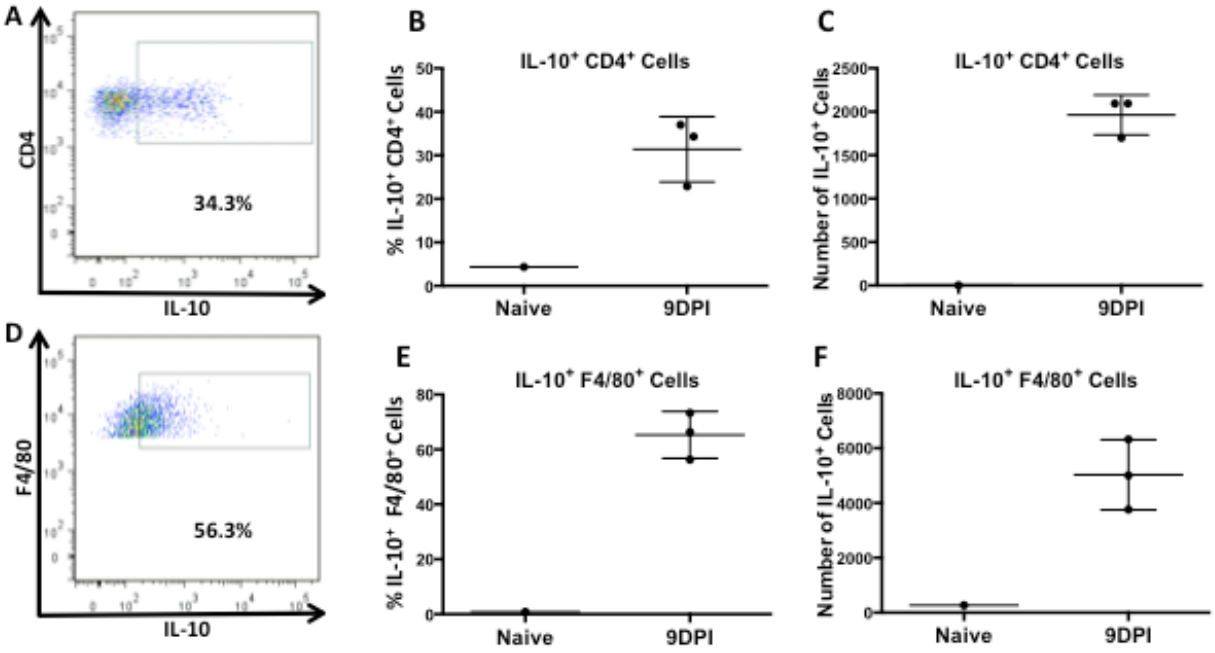

Figure 12. A large proportion of F4/80⁺ cells present in the TG during primary lytic infection express IL-10.

B6.129S6-Il10^{tmvFlv}/J mice were bilaterally infected with 1X10^5 PFU HSV-1-KOS/eye. At 9 DPI TGs were harvested and processed into single cell suspensions before being stained for CD45, CD4, and F4/80 and IL-10-GFP expression was assessed by flow cytometry. Flow cytometry plots indicating IL-10-GFP expression in CD4⁺ cells (A) and F4/80⁺ cells (D) from the TGs of infected mice are shown. The proportion (B & E) and number (C & F) of CD4⁺ cells (B & C) and F4/80⁺ cells (E & F) expressing IL-10-GFP in naïve and HSV-1-infected mice are indicated. (G) and (H) show the relative IL-10-GFP MFI levels in CD4⁺ and F4/80⁺ cells in naïve and HSV-1-infected mice. All flow cytometry plots shown were previously gated CD45. All bars represent the mean ± SEM of cell type indicated. Data is representative of a single experiment (N=3 for infected samples and 1 for naïve sample).

Although depletion of $CD4^+$ T cells does have a robust effect on non-gB $CD8^+$ T cell numbers and also results in a pattern of increased GrzB expression in $CD8^+$ T cells, we cannot eliminate the possibility that other cell populations in the latently infected TG express IL-10 to modulate $CD8^+$ T cell function. An $F4/80^+$ population is present in the TGs of B6.129S6-$Il10^{tmlFlv}$/J mice at 9DPI that is greater in number and proportional expression of IL-10 than $CD4^+$ T cells **(Figure 12)**. Given that $F4/80^+$ cells are expressing IL-10 in high numbers in the TG during acute infection, it is possible that an $F4/80^+$ $IL\text{-}10^+$ population is present in the TG following the establishment of latent HSV-1.

6.4 DISCUSSION AND FUTURE DIRECTIONS

Since we had observed 20% of $CD4^+$ T cells expressing IL-10 and blocking IL-10R resulted in an increase in the number and functionality of subdominant epitope-specific $CD8^+$ T cells [45], we anticipated a similar result upon depletion of $CD4^+$ T cells. However, our initial study examining the effects of CD4 depletion on $CD8^+$ T cell functions indicated that there was no requirement for $CD4^+$ T cells in the regulation of $CD8^+$ T cell function **(Figure 9).** This indicated one of two things: 1) $CD4^+$ T cells are not the primary contributors of this cytokine or 2) an additional immune cell population is capable of up-regulating IL-10 expression in the absence of $CD4^+$ T cells. However, given the low T cell numbers in this experiment, it is also possible that this is not representative of a typical immune response to HSV-1 and $CD8^+$ T cell response to a lack of CD4 presence. As such, we repeated the depletion experiment **(Figures 10 and 11)** and examined IL-10 expression in cell populations other than $CD4^+$ T cells within the TG following HSV-1 infection **(Figure 12).**

When we repeated the CD4 depletion experiment, the TG-infiltrating T cell numbers were similar to those typically observed in our studies. The effect of $CD4^+$ T cell depletion on subdominant epitope-specific $CD8^+$ T cells in this experiment was similar to the effect we had previously seen upon IL-10R blockade **(Figure 10)** [45], causing us to speculate that $CD4^+$ T cells may, in fact, be the key producers of the IL-10 that downregulates subdominant epitope-specific $CD8^+$ T cell function in the TG during latent HSV-1 infection. $CD4^+$ T cell-secreted IL-10 is known to dampen pro-inflammatory immune responses during numerous persistent pathogenic infections including HCMV, LCMV, *plasmodium*, and *toxoplasma gondii* [97-101]. Therefore, it is feasible that this also occurs during HSV-1 latency in the TG. The $CD4^+$ T cells that were remaining in TGs following depletion showed reduced IL-10 expression **(Figure 11)**, indicating a potential reduction in their ability to moderate $CD8^+$ T cell activity through expression of this cytokine. This would also help to explain the increase in CD8 number and function even though $CD4^+$ T cells positive for IL-10 remain in the TG following depletion.

Further studies are needed to assess the degree to which $CD4^+$ T cell-secreted IL-10 regulates HSV-1 latency and reactivation. These will likely include examining the reactivation frequencies of TGs depleted of $CD4^+$ T cells concurrent with assessing IL-10 transcript or protein levels in these cultures. If these studies indicate a significant effect of $CD4^+$ T cell-secreted IL-10 on HSV-1 reactivation, we will then examine means by which to reduce $CD4^+$ T cell numbers or IL-10 expression within the latently infected TG. Levels of IL-10 production in additional immune cell populations within the latently infected TG in the absence of $CD4^+$ T cells must also be determined to confirm that there is not additional IL-10 production in the TG.

When we examined IL-10 expression in the TGs of IL-10-GFP reporter mice at 9 DPI, we did observe a large F4/80$^+$ cell population in the TG that was greater in size than the CD4$^+$ T cell population **(Figure 12).** We had not observed this population in our initial study examining the sources of IL-10 in the TG during latency [45], but it is possible that this F4/80$^+$ IL-10$^+$ cell population is an infiltrating or tissue-resident macrophage population and is also present in latent HSV-1 infection. Macrophage populations are known to traffic to peripheral tissues and sites of immune activation to mediate primary immune responses in these areas. They are also known for their secretion of IL-10 to dampen the inflammatory response during chronic models of infection and cancer [102, 103]. Thus, this F4/80$^+$ cell population, if present in the TG during latent HSV-1 infection, may serve as an additional source of IL-10 that regulates CD8$^+$ T cell function thereby mediating HSV-1 latency and reactivation.

We attempted to examine IL-10 expression in additional cell types, including in F4/80$^+$ cells, during latent HSV-1 infection in our IL-10-GFP reporter mice. However, this experiment yielded inconclusive data and must be repeated. If F4/80$^+$ cells are present and express IL-1, in the TG during latent HSV-1 infection, we will further define this population by assessing surface marker expression of Ly6C, Ly6G, CD11b, and CD11c. These markers are known to be variably expressed on F4/80$^+$ macrophages, neutrophils, and DCs and can be used to differentiate the populations by flow cytometry and fluorescent imaging [95, 104-106]. We will then attempt to assess the effects this population has on CD8$^+$ T cell function and HSV-1 latency as we have done here or propose to do with CD4$^+$ T cells. Finally, we will assess the effects of concurrent depletion of CD4$^+$ and F4/80$^+$ cells on CD8$^+$

T cell function and HSV-1 reactivation to determine if the effect of dual depletion is equivalent to that of $CD4^+$ T or $F4/80^+$ cell depletion alone.

7.0 SUMMARY AND CONCLUSION

We set out to define the answers to three specific questions, as outlined in Chapter 2. In our first aim we wished to define the HSV-1-specific $CD4^+$ T cell repertoire in the TGs of B6 mice following HSV-1 infection. Here we have shown that a portion of $CD4^+$ T cells in the TG during latent HSV-1 infection specifically recognize HSV-1 in the presence of professional APCs *ex vivo* and respond by secreting IFN-γ and IL-10. We have also shown that a small but significant percentage of $CD4^+$ T cells present in the spleens of HSV-1-infected mice specifically recognize HSV-1-gD peptide and secrete IFN-γ in response. However, we have yet to define the full spectrum of HSV-1 epitopes that $CD4^+$ T cells present in the spleens and TGs of infected mice recognize and will continue our studies to define the full HSV-1-specific $CD4^+$ T cell repertoire. This information will aid future investigators in examining the mechanisms of immune control of latent HSV-1 infection and may also shed light on HSV-specific $CD4^+$ T cell populations that can be targeted for manipulation to mediate HSV-1 latency in the TG.

In Aim 2, we set out to define the interactions of $CD4^+$ T cells with other cell types, specifically with $MHCII^+$ cell populations, in the latently infeced TG and wished to determine if $CD4^+$ T cells isolated from TGs harboring latent HSV-1 infection are stimulated by these cells in the presence of HSV-1 antigen *ex vivo*. We found that $CD4^+$ T cells in the TG are apposed to $F4/80^+$ cells as well as neurons and possibly satellite glial cells associated with neurons. We have yet to assess MHCII expression within the TG during latent HSV-1

infection and thus, cannot say if these cell types have the potential to serve as APCs to $CD4^+$ T cells in this tissue. We will assess MHCII expression and will examine the ability of any $MHCII^+$ populations present in the TG to activate $CD4^+$ T cells *ex vivo* in the presence of HSV-1 antigen. Identification of a TG-resident APC population capable of stimulating $CD4^+$ T cells may allow for the assessment of $CD4^+$ T cell activation, function, and exhaution levels within the TG. These studies will better define the functions of $CD4^+$ T cell population within the TG and may also yield insight into means by which $CD4^+$ T cells and APC populations within the HSV-1-infected TG can be activated or inactivated to modulate immune activity, particularly that of $CD8^+$ T cells, to prevent HSV-1 reactivation.

In our final aim, we wanted to follow up on two earlier observations: 1) that $CD4^+$ T cells present in the TG produce IL-10 and appear to be the sole population to do so and 2) that blocking IL-10 signaling bolsters subdominant epitope-specific $CD8^+$ T cell numbers and functionality and reduces HSV-1 reactivation frequency [45]. In the current study, $CD4^+$ T cell depletion results in an increase in non gB-specific $CD8^+$ T cell numbers and an increase in GrzB expression in both gB-specific and non gB-specific $CD8^+$ T cell populations, an expected result that is similar to that seen with IL-10R blockade [45]. This indicates that $CD4^+$ T cells are the dominant IL-10 producer in the TG and that they reduce non gB-specific $CD8^+$ T cell functionality through IL-10 signaling. Reduced non-gB $CD8^+$ T cell functionality results in an increased rate of HSV-1 reactivation and viral particle production [45]. Additional studies to confirm these findings and further define the molecular mechanisms controlling both $CD4^+$ expression of and $CD8^+$ T cell responses to IL-10 may identify mechanisms that can be manipulated to bolster protective responses and prevent HSV-1 reactivation while minimizing immune-mediated pathology. $F4/80^+$ cells also

produce IL-10 in the TG during acute infection, but it remains unclear if these cells express IL-10 in the TG upon the establishment of latent infection and this will be examined in future studies as was conducted with $CD4^+$ T cells.

Collectively, these studies have and will continue to provide new insights into $CD4^+$ T cell activity in latent HSV-1 infection and may result in the generation of new tools such as HSV-1:MHCII tetramers by which to examine $CD4^+$ T cells in HSV-1 infection. Furthermore, the information gained from these studies may aid in the development of therapeutic vaccines that modulate $CD4^+$ as well as $CD8^+$ T cell function in the TG to promote latency and prevent HSV-1 reactivation events and the pathologies associated with them.

BIBLIOGRAPHY

1. Schillinger J, Xu F, Sternberg MR, Armstrong GL, Lee FK, Namias AJ, McQuillan GM, Louis ME, Markowitz LE. 2004. National seroprevalance and trends in Herpes simplex virus type 1 in the United States, 1976-1994. *Sex Trans Dis* 31:753-760.

2. Toma HS, Murina AT, Areaux RG, Neumann DM, Bhattacharjee PS, Foster TP, Kaufman HE, Hill JM. 2009. Ocular HSV-1 latency, reactivation and recurrent disease. *Sem in Ophth* 23: 249-273.

3. Xu F, Sternberg MR, Kottiri BJ, McQuillan GM, Lee FK, Nahmias AJ, Berman SM, Markowitz LE. 2006. Trends in Herpes simplex virus type I and type II seroprevalence in the United States. *J Am Med Assoc* 296: 964-73.

4. Smith JA, Robinson NJ. 2002. Age-Specific prevalence of infection with Herpes simplex virus types 2 and 1: A global review. *J Infect Dis* 186: S3-S28.

5. Sauerbrei A, Schmitt S, Scheper T, Brandstadt A, Saschenbrecker S, Motz M, Soutschek E, Wutzler P. 2011. Seroprevalence of herpes simplex virus type 1 and type 2 in Thuringia, Germany, 1999 to 2006. *Euro Surveill* PMID: 22085620.

6. Brazzale AG, Russell DB, Cunningham AL, Taylor J, McBride WJ. 2010. Seroprevalence of herpes simplex virus type 1 and type 2 among the Indigenous population of Cape York, Far North Queensland, Australia. *Sex Health* 7: 453-9.

7. Liesegang TJ. 2001. Herpes simplex virus epidemiology and ocular importance. *Cornea* 20: 1-13.

8. Farooq AV, Shukla D. 2012. Herpes simplex epithelial and stromal keratitis: an epidemiologic update. *Surv of Ophth* 57: 448-62.

9. Knipe DM, Howley PM, Griffin DE, Lamb RA, Martin MA, Roizman B, Strauss SE. 2007. *Fields Virology*. Lippincott Williams & Wilkins, Hagerstown, MD.

10. Roizman B, Knipe, DM. 2001. Herpes simplex viruses and their replication; *Fields Virology*; Knipe DM, Howley PM, Straus SE, Martin MA, editor. Philadelphia: Lippincott - Raven. pp. 2399 – 2459.

11. Whitley R. 1996. *Fields Virology*; Knipe DM, Griffin D, Lamb R, Martin M, Straus S, editor. Philadelphia: Lippincott, Williams & Wilkins. pp. 2297-2342.

12. Fukuda M, Deai T, Higaki S, Hayashi K, Shimomura Y. 2008. Presence of a large amount of herpes simplex virus genome in tear fluid of herpetic stromal keratitis and persistent epithelial defect patients. *Semin Ophthl* 23: 217-20.

13. Jones BR. 1958. The clinical features of viral keratitis and a concept of their pathogenesis. *Proc R Soc Med* 51: 917-24.

14. Kumaraguru U, Davis I, Rouse BT. 1999. Chemokines and ocular pathology caused by corneal infection with herpes simplex virus. *J Neurovirol*. 5: 42-7.

15. Spurney RV, Rosenthal MS. 1972. Ultraviolet-induced recurrent herpes simplex virus keratitis. *Am J Ophthal* 73: 609-10.

16. Sawtell NM, Thompson RL. 1992. Rapid in vivo reactivation of herpes simplex virus in latently infected murine ganglionic neurons after transient hyperthermia. *J Virol* 66: 2150-56.

17. Padgett DA, Sheridan JF, Dorne J, Berston GG, Candelora J, Glaser R. 1998. Social stress and the reactivation of latent herpes simplex virus type 1. *Proc Nat Acad Sci USA* 95: 7231-35.

18. Freeman ML, Sheridan BS, Bonneau RH, Hendrcks RL. 2007. Psychological stress compromises $CD8^+$ T cell control of latent herpes virus type 1 infections. *J Immunol* 179: 322-8.

19. Cherpes TL, Busch JL, Sheridan BS, Harvey SAK, and Hendricks RL. 2008. Medroxyprogesterone acetate inhibits $CD8^+$ T cell viral-specific effector function and induced herpes simplex virus type 1 reactivation. *J Immunol* 181: 969-75.

20. Chentoufi AA, Kritzer E, Yu DM, Nesburn AB, BenMohamed L. 2012. Towards a rational design of an asymptomatic clinical Herpes vaccine: The old, the new, and the unknown. *Clin Dev immunol* PMCID: PMC3324142.

21. Coleman JL, Shukla D. 2013. Recent Advances in vaccine development for Herpes simplex types I and II. *Hum Vaccin Immunother* 9: 729-35.

22. Whitley RJ. 1985. Therapy for human herpesvirus infections. A perspective. *Ala J Med Sci* 22: 193-207.

23. Koelle, DM. & Corey L. 2003. Recent progress in herpes simplex virus immunobiology and vaccine research. *Clin Microbiol Rev.* 16:96-113.

24. Herold BC, WuDunn D, Soltys N, Spear PG. 1991. Glycoprotein C of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. *J Virol* 65: 1090-1098.

25. Shieh MT, Spear PG. 1994. Herpesvirus-induced cell fusion that is dependent on cell surface heparan sulfate or soluble heparin. *J Virol* 68: 1224-1228.

26. Turner A, Bruun B, Minson T, Browne H. 1998. Glycoproteins gB, gD, and gHgL of herpes simplex virus type 1 are necessary and sufficient to mediate membrane fusion in a Cos cell transfection system. *J Virol* 72: 873-875.

27. Jackson SA, DeLuca NA. 2003. Relationship of herpes simplex virus genome configuration to productive and persistent infections. *Proc Nat Acad Sci USA* 100: 7871-76.

28. Deshmane SL, Fraser NW. 1989. During latency, herpes simplex virus type 1 DNA is associated with nucleosomes in a chromatin structure. *J Virol* 63: 943-9.

29. Kubat NJ, Amelio AL, Giordani NV, Bloom DC. 2004. The Herpes Simplex Virus Type 1 Latency-Associated Transcript (LAT) Enhancer/rcr Is Hyperacetylated during Latency Independently of LAT Transcription. *J Virol* 78: 12508-18.

30. Steiner I, Spivack JG, O'Boyle DR, Lavi E, and Fraser NW. 1988. Latent herpes simplex virus type 1 transcription in human trigeminal ganglia. *J Virol* 62:3493-96.

31. Krause PR, Croen KD, Straus SE, Ostrove JM. 1988. Detection and preliminary characterization of herpes simplex virus type 1 transcripts in latently infected human trigeminal ganglia. *J Virol* 62: 4819-23.

32. Stevens JG, Haarr L, Porter DD, Cook ML, Wagner EK. 1988. Prominence of the herpes simplex virus latency-associated transcript in trigeminal ganglia from seropositive humans. *J Inf Dis* 158: 117-23.

33. Kramer MF, Chen SH, Knipe DM & Coen DM. 1998. Accumulation of viral transcripts and DNA during establishment of latency by herpes simplex virus. *J Virol* 72: 1177-85.

34. Kramer MF, Coen DM. 1995. Quantification of transcripts from the ICP4 and thymidine kinase genes in mouse ganglia latently infected with herpes simplex virus. *J Virol* 69: 1389-99.

35. Derfuss T, Segerer S, Herberger S, Sinicina I, Hufner K, Ebelt K, Knaus HG, Steiner I, *et al*. 2007. Presence of HSV-1 immediate early genes and clonally expanded T-cells with a memory effector phenotype in human trigeminal ganglia. *Brain Pathol* 17:389-98.

36. Held K, Junker A, Dornmair K, Meinl E, Sinicina I, Brandt T, Theil D, Derfuss T. 2011. Expression of herpes simplex virus 1-encoded microRNAs in human trigeminal ganglia and thie relation to local T-cell infiltrates. *J Virol* 85: 9680-85.

37. Chen SH, Kramer MF, Schaffer PA, Coen DM. 1997. A viral function represses accumulation of transcripts from productive-cycle genes in mouse ganglia latently infected with herpes simplex virus. *J Virol* 71: 5878-84.

38. vanVelzen M, Jing L, Osterhaus A, Sette A, Koelle DM, Verjans GM. 2013. Local CD4 & CD8 T-cell reactivity to HSV-1 antigens documents broad viral protein expression and immune competence in latently infected human trigeminal ganglia. *PlosPath* 9(8): e1003547.

39. Feldman LT, Ellison AR, Voytek CC, Yang L, Krause P, Margolis TP. 2002. Spontaneous molecular reactivation of herpes simplex virus type 1 latency in mice. *Proc Nat Acad Sci* 99: 978-83.

40. St. Leger AJ, Peters B, Sidney J, Sette A, Hendricks RL. 2011. Defining the Herpes Simplex Virus-specific $CD8^+$ T cell repertoire in C57BL/6 mice. *J Immunol* 186: 3927-33.

41. Decman V, Kinchington PR, Harvey SAK, Hendricks RL. 2005. Gamma interferon can block Herpes Simplex Virus 1 reactivation from latency, even in the presence of late gene expression. *J Virol* 79: 10339-47.

42. Knickelbein JE, Khanna KM, Yee MB, Baty CJ, Kinchington PR, Hendricks RL. 2009. Noncytotoxic lytic granule-mediated inhibition of HSV-1 reactivation from neuronal latency. *Science* 322: 268-71.

43. Liu T, Tang Q, Hendricks RL. 1996. Inflammatory infiltration of the trigeminal ganglion after Herpes Simplex Virus Type 1 corneal infection. *J Virol* 70: 264-71.

44. Liu T, Khanna KM, Carriere BN, Hendricks RL. 2001. Gamma interferon can prevent Herpes Simplex Virus Type I reactivation from latency in sensory neurons. *J Virol* 75: 11178-84.

45. St. Leger AJ, Jeon S, Hendricks RL. 2013. Broadening the repertoire of functional Herpes Simplex Virus Type I-specific $CD8^+$ T cells reduces viral reactivation from latency in sensory ganglia. *J Immunol* 191: 2258-65.

46. Theil D, Derfuss T, Paripovic I, Herberger S, Meinl E, Schueler O, Strupp M, Arbusow V, Brandt T. 2003. Latent Herpesvirus infection in human trigeminal ganglia causes chronic immune response. *Am J Pathol* 163: 2179-84.

47. Nakanishi Y, Lu B, Gerard C, Iwasaki A. 2009. CTL mobilization to virus-infection requires $CD4^+$ T cell help. *Nature* 462: 510-13.

48. Williams MA, Tyznik AJ, Bevan MJ. 2006. Interleukin-2 signals during priming are required for secondary expansion of $CD8^+$ memory T cells. *Nature* 441: 890-93.

49. Wilson EB, Livingstone AM, 2008. $CD4^+$ T cell-derived IL-2 is essential for help-dependent primary $CD8^+$ T cell responses. *J Immuol* 181: 7445-48.

50. Smith CM, Wilson NS, Waithman J, Villandangos JA, Carbone FR, Heath WA, Belz, GT. 2004. Cognate $CD4^+$ T cell licensing of dendritic cells in $CD8^+$ T cell immunity. *Nat Immunol* 5(11): 1143-48.

51. Bennett SR, Carbone FR, Karamalis F, Flavell RA, Miller JF, Heath WR. 1998. Help for cytotoxic-T-cell responses is mediated by CD40 signaling. *Nature* 393: 478-80.

52. Frohlich A, Kisielow J, Schmitz I, Freigang S, Shamshiev AT, Weber J, Marsland BJ, Oxenius A, Kopf M. 2009. IL-21R on T cells is critical for sustained functionality and control of chronic viral infection. *Science* 324: 1576-80.

53. Elasser H, Sauer K, Brooks DG. 2009. IL-21 is required to control chronic viral infection. *Science* 324: 1569-72.

54. Yi JS, Du M, Zajac AJ. 2009. A vital role for interleukin-21 in the control of a chronic viral infection. *Science* 324: 1572-76.

55. Stuller KA, Cush SS, Flano E. 2010. Persistent γ-Herpesvirus infection induces a CD4 T cell response containing functionally distinct effector populations. *J Immunol* 184: 3850-56.

56. Stuller KA, Flano E. 2009. CD4 T cells mediate killing during persistent gammaherpesvirus 68 infection. *Virology* 83(9): 4700-03.

57. Zajac AJ, Quinn DG, Cohen PL, Frelinger JA. 1996. Fas-dependent $CD4^+$ cytotoxic T-cell-mediated pathogenesis during virus infection. *Proc Nat Acad Sci USA* 93(25): 14730-35.

58. Gebhardt T, Whitney PG, Zaid A, Mackay LK, Brooks AG, Heath WR, Carbone FR, Mueller, SN. 2011. Different patterns of peripheral migration by memory $CD4^+$ and $CD8^+$ T cells. 2011. *Nature* 477: 216-21.

59. Asea A, Rehli M, Kabingu E, Boch JA, Bare O, Auron PE, Stevenson MA, Calderwood SK. 2002. Novel signal transduction pathway utilized by extracellular HSP70. *J Biol Chemistry* 277(17): 15028-34.

60. Sarangi PP, Kim B, Kurt-Jones E, Rouse BT. 2007. Innate recognition network driving herpes simplex virus-induced corneal immunopathology: role of the toll pathway in early inflammatory events in stromal keratitis. *J Virol* 81: 11128-38.

61. Kassim SH, Rajasagi NK, Zhao X, Chervenak R, Jennings SR. 2006. In vivo ablation of CD11c-positive dendritic cells increases susceptibility to Herpes Simplex Virus Type 1 infection and diminishes NK and T-cell Responses. *J Virol* 80(8): 3985-93.

62. Smith CM, Belz GT, Wilson NS, Villandangos JA, Shortman K, Carbone FR, Heath WR. 2003. Conventional $CD8\alpha^+$ dendritic cells are preferentially involved in CTL priming after footpad infection with Herpes Simplex Virus-1. *J Immunol* 170: 4437-40.

63. Bedoui S, Whitney PG, Waithman J, Eidsmo L, Wakim L, Caminschi I, Allan RS, Wojtasiak M, Shortman K, Carbone FR, Brooks AG, Heath WR. 2009. Cross-presentation of viral and self antigens by skin-derived $CD103^+$ dendritic cells. *Nat Immunol* 10(5): 488-95.

64. del Rio ML, Rodriguez-Barbosa, J, Kremmer E, Forster R. 2007. $CD103^-$ and $CD103^+$ bronchial lymph node dendritic cells are specialized in presenting and cross-presenting innocuous antigen to $CD4^+$ and $CD8^+$ T cells. *J Immunol* 178: 6861-66.

65. Zhao X, Deak E, Soderberg K, Linehan M, Spezzano D, Zhu J, Knipe DM, Iwasaki A. 2003. Vaginal submucosal dendritic cells, but not Langerhans cells, induce proliferative Th1 responses to Herpes Simplex Virus-2. *J Exp Med* 197: 153-62.

66. Lee HK, Zamora M, Linehan MM, Iijima N, Gonzalez D, Haberman A, Iwasaki A. 2009. Differential roles of migratory and resident DCs in T cell priming after mucosal or skin HSV-1 infection. *J Exp Med* 206(2): 359-70.

67. McLachlan JB, Catron DM, Moon JJ, Jenkins MK. 2009. Dendritic cell antigen presentation drives simultaneous cytokine production by effector and regulatory T cells in inflamed skin. *Immunity* 30: 277-88.

68. Iijima N, Matthei LM, Iwasaki A. 2011. Recruited inflammatory monocytes stimulate antiviral Th1 immunity in infected tissues. *Proc Nat Acad Sci* 108(1): 284-89.

69. Kanagat S, Thomas L, Gangappa S, Babu JS, Rouse BT. 1996. Herpes simplex virus type I mediated up-regulation of IL-12 (p40) mRNA expression. Implications in immunopatho-genesis and protection. *J Immunol* 156:1110-16.

70. Malmgaard L, Paludan SR, Mogensen SC, Ellerman-Eriksen S. 2000. Herpes simplex virus Type 2 induces secretion of IL-12 by macrophages through a mechanism involving NF-κB. *J Gen Virol* 81: 3011-3020.

71. Cheng H, Tumpey TM, Staats HF, van Rooijen N, Oakes JE, Lausch RN. 2000. Role of macrophages in restricting Herpes Simplex Virus Type I growth after ocular infection. *Invest Ophthalmol Vis Sci* 41:1402-09.

72. Shimeld C, Whiteland JL, Nicholls SM, Gringield E, Easty DL, Ga H, Hill TJ. 1995. Immune cell infiltration and persistence in the mouse trigeminal ganglion after infection of the cornea with herpres simplex virus type I. *J Neuroimm* 61: 7-16.

73. Pereira RA, Tscharke A, Simmons A. 1994. Upregulation of class I histocompatibility complex gene expression in primary sensory neurons, satellite cells, and Schwann cells of mice in response to acute but not latent herpes simplex virus infection in vivo. *J Exp* Med 180: 841-50.

74. Baetas-da-Cruz W, Alves L, Pessolani MCV, Barbosa HS, Regnier-Vigouroux A, Corte-Real-S, Cavalcante LA. 2009. Schwann cells express macrophage mannose receptor and MHC class II.Do they have a role in antigen presentation? *J Periph Nerv Sys* 14:84-92.

75. Hirsch MR, Wietzerbin J, Pierres M, Goridis C. 1983. Expression of Ia antigens by cultured astrocytes treated with gamma-interferon. *Neurosci Lett* 41: 199-204.

76. Scriba M. 1975. Herpes simplex virus infection in guinea pigs: an animal model for studying latent and recurrent herpes simplex virus infection. *Infect Immunol* 12(1): 162-65.

77. Nesburn AB, Green MT, Walker B. 1977. Reliable in vivo model for latent herpes simplex virus reactivation with peripheral virus shedding. *Infect Immunol* 15(3): 772-75.

78. Webre JM, Hill JM, Nolan NM, Clement C, McFerrin HE, Bhattacharjee PS, Hsia V, Neumann DM, *et al* 2012. Rabbit and mouse models of HSV-1 latency, reactivation, and recurrent eye diseases. *J Biomed Biotech* eID: 612316.

79. Smith KO. 1964. Relationship between the envelope and the infectivity of herpes simplex virus. *Proc Soc Exp Biol Med* 115: 814-16.

80. Himmelein S, St. Leger AJ, Knickelbein JE, Rowe AM, Freeman ML, Hendricks RL. 2011. Circulating herpes simplex type 1 (HSV-1)-specific $CD8^+$ T cells do not access HSV-1 latently infected trigeminal ganglia. *Herpesviridae* 2:5.

81. Mikloska Z, Kesson AM, Penfold, ME, Cunningham AL. 1996. Herpes simplex virus protein targets for CD4 and CD8 lymphocyte toxicity in cultured epidermal keratinocytes treated with interferon-γ. *J Infect Dis* 173: 7-17.

82. Mikloska Z, Cunningham AL. 1998. Herpes simplex virus type 1 glycoproteins gB, gC, and gD are major targets for CD4 T-lymphocyte cytotoxicity in HLA-DR expressing human epidermal keratinocytes. *J Gen Virol* 79: 353-61.

83. Verjans GM, Remeijer L, van Binnendijk RS, Cornelissen JG, Volker-Dieben HJ, Baarsma SG *et al*. 1998. Identification and characterization of herpes simplex virus-specific $CD4^+$ T cells in corneas of herpetic stromal keratitis patients. *J Infect Dis* 177: 484-88.

84. Koelle DM, Reymond SN, Chen H, Kwok WW, McClurkan C, Gyaltsong T, Petersdorf EW *et al.* 2000. Tegument-specific, virus-reactive CD4 T cells localize to the cornea in herpes simplex virus interstitial keratitis in humans. *J Virol* 74: 10930-38.

85. Kim M, Taylor J Sidney J, Mikloska Z, Bodsworth N, Lagios K, Dunckley H, Byth-Wilson K, *et al.* 2008. Immunodominant epitopes in Herpes Simplex Virus Type 2 Glycoprotein D are recognized by CD4 lymphocytes from both HSV-1 and HSV-1 seropositive subjects. *J immunol* 181: 6604-15.

86. Williams MA, Ravkov EV, Bevan MJ. 2008. Rapid culling of the CD4+ T cell repertoire in the transition from effector to memory. *Immunity* 28:533-45.

87. vanVelzen M, Laman, JD, KleinJan A, Poot A, Osterhaus ADME, Verjans GM. 2009. Neuron-interacting satellite glial cells in human trigeminal ganglia have an APC phenotype. *J Immunol* 183: 2456-61.

88. Soriano P. 199. Generalized LacZ expression with thr ROSA26 Cre reporter strain. *Nat Genet* 21: 70-1.

89. Proenca J, Coleman HM, Connor V, Wnton DJ, Efstathiou S. 2008. A historical analysis of herpes simplex virus promoter activation *in vivo* reveals distinct populations of latently infected neurones. *J Gen Virol* 89: 2965-74.

90. Proenca J, Coleman HM, Nicoll MP, Connor V, Preston CM, Arthur J, Efstathiou S. 2011. An investigation of herpes simplex virus promoter activity compatible with latency establishment reveals VP16-independent activation of immediate-early promoters in sensory neurons. *J Gen Virol* 92: 2575-85.

91. Maitreyi S, Milho R, May JS, Nicoll MP, Efstathiou S, Stevenson PG. 2013. Herpes simplex virus 1 targets the murine olfactory neuroepithelium for host entry. *J Virol* 87: 10477-88.

92. Sheridan BS, Cherpes TL, Urban J, Kalinski P, Hendricks RL. 2009. Reevaluating the CD8 T-cell response to herpes simplex virus type 1: involvement of CD8 T cells reactive to subdominant epitopes. *J Virol* 83: 2237-45.

93. Frank GM, Lepisto AJ, Freeman ML, Sheridan BS, Cherpes TL, Hendricks RL. 2010. Early CD4+ T cell help prevents partial CD8+ T cell exhaustion and promotes maintenance of herpes simplex virus type 1 latency. *J Immunol* 184: 277-86.

94. Jeon S, St Leger A, Cherpes TL, Sheridan BS, Hendricks RL. 2013. PD-L1/B7-H1 regulates the survival but not the function of CD8+ T cells in latently infected trigeminal ganglia. *J Immunol* 190: 6277-86.

95. Rose S, Misharin A, Perlman H. 2012. A novel Ly6C/Ly6G-based strategy to analyze the mouse splenic myeloid compartment. *Cytometry A* 81: 343-50.

96. Macleod B, Bedoui S, Hor JL, Mueller SN, Russell TA, Hollett NA, Heath WR, Tscharke DC, Brooks, AG, Gebhardt T. 2014. Distinct APC subtypes drive spatially segregated CD4+ and CD8+ T-cell effector activity during skin infection with HSV-1. *PloS Pathog* 10: e1004303.

97. Jankovic D, Kullberg MC, Feng CG, Goldszmid RS, Collazo CM, Wilson M, Wynn TA, Kamanaka M, *et al.* 2007. Converntional T-bet+ FoxP3- Th1 cells are the major source of

host-protective regulatory IL-10 during intracellular protozoan infection. *J Exp Med* 204: 273-83.

98. Puntambekar SS, Bergmann CC, Savarin C, Karp CL, Phares TW, Parra GI, Hinton DR, Stohlman SA. 2011. Shifting hierarchies on interleukin-10-producing T cell populations in the central nervous system during acute and persistent viral encephalomyelitits. *J Virol* 85: 6702-13.

99. Frietas do Rosario AP, Lamb T, Spence P, Stephens R, Lang A, Roers A, Muller W, O'Garra A, Langhorne J. 2012. IL-27 promotes Il-10 production by effector Th1 $CD4^+$ T cells; a critical mechanism for protection from severe immunopathology during malaria infection. *J Immunol* 188:1178-90.

100. Mason GM, Jackson S, Okecha G, Poole E, Sissons JGP, Sinclair J, Wills MR. 2013. Human cytomegalovirus latency-associated proteins elicit immune-suppressive IL-10 producing $CD4^+$ T cells. *PLOSpath*: e1003635.

101. Parish IA, Marshall HD, Staron MM, Lang PA, Brustle A, Chen JH, Cui W, Tsui YC, Perry C, *et al*. 2014. Chronic viral infection promotes sustained Th1-derived immunoregulatory IL-10 via BLIMP-1. *J Clin Invest* 124: 3455-68.

102. Mosser DM, Zhang X. 2008. Interleukin-10: new perspectives on an old cytokine. *Immunol Rev* 226: 205-18.

103. Richter K, Perriard G, Behrendt R, Schwender RA, Sexl V, Dunn R, Kamanaka M, Flavell RA, *et al*. 2013. Macrophage and T cell produced IL-10 promotes viral chronicity. *PLOSPath*: e1003735.

104. Austyn JM, Gordon S. 1981. F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *Eur J Immunol* 11: 805-15.

105. Mosser DM, Edwards JP. 2008. Exploring the full spectrum of macrophage activation. *Nat Rev Immunol* 8:958-69.

106. Yona S, Jung S. 2010. Monocytes: subsets, origins, fates and functions. *Curr Opin Hematol* 17: 53-59.